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The biosynthesis of pyrimidines by mutants of Aerobacter aerogenes

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NOTE TO USERS

THE BIOSYNTHESIS OF PYRIMIDINES BY MUTANTS OF AEROBACTER AEROGENES

by

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INTRODUCTION

During the past ten years, there has been a widespread development of the use of metabolically blocked organisms for the elucidation of biosynthetic reaction sequences. These organisms may possess an endogenous block, occasioned by mutation, or an exogenous block, produced by the addition of a chemical inhibitor. In either case, the formation of a normal intermediate or end product is prevented, and the intermediate which precedes the block often accumulates. In experiments involving metabolically blocked organisms, various postulated intermediates are added in an attempt to remove the effect of the enzymatic block. Through the study of accumulated product(s) and the ability of added compounds to re-establish normal metabolic functions, considerable evidence can be obtained regarding the pathways of biosynthesis.

Although the biological formation of pyrimidines in different organisms has been studied by various investigators, relatively few workers have been concerned with pyrimidine biosynthesis in bacteria. A study of this phase of metabolism in Aerobacter aerogenes therefore seemed warranted.

In the course of this investigation, several types of pyrimidine auxotrophs of Aerobacter aerogenes have been produced. These mutants appear to be blocked at different points in the pathway of pyrimidine biosynthesis.

This is the first investigation to employ artificially induced bacterial mutants for the analysis of pyrimidine biosynthesis in the living organism.

REVIEW OF LITERATURE

General Pyrimidine Metabolism Studies

Early excretion studies

The first pioneer in the field of pyrimidine metabolism was Steudel (1901) who reported an increased excretion of urea by dogs which had been fed thymine.

Mendel and Myers (1910) studied the fate of thymine, cytosine, and uracil when introduced into man and various animals in different ways. They found that pyrimidine ingestion did not appear to contribute to the formation of the purines eliminated in the urine. Creatinine metabolism was unaffected. The pyrimidines appeared more stable in metabolism than the purines, which were readily transformed. Contrary to the finding of Steudel, they noted no marked increase in urea-ammonia output after the feeding of pyrimidine bases. However, they observed increased elimination of other nitrogenous compounds. When nucleic acid was administered, no pyrimidine bases were detected in normal urine, but after the feeding or injection of the free pyrimidine bases, these substances were recovered unchanged in the urine. These findings would indicate that the pyrimidine bases are not metabolized to any great extent except in combination with other nucleic acid components.

Wilson (1920, 1923) used pyrimidines as the only source of nitrogen

for rabbits. Increased urea excretion was noted. When uridine was fed, considerable amounts of uracil were excreted in the urine. He found that increasing quantities of uracil appeared in the urine as simpler complexes containing uracil were fed. This observation was similar to that of Mendel and Myers (1910). Wilson concluded that in the metabolism of yeast nucleic acid, the pyrimidine portion is attacked before the free pyrimidine base is liberated, and even before the pyrimidine nucleotide has been hydrolyzed to form the nucleoside. He believed that the pyrimidine base could be broken down and its nitrogen converted into urea even though it existed in the nucleotide or more complex state. Uracil appeared to be quantitatively excreted unchanged, but uridine and uridylic acid were metabolized.

Deuel (1924) studied pyrimidine metabolism in dogs and man. He found that thymine and uracil were largely excreted unchanged, unless fed in small divided doses over a period of days. When fed in small amounts, an increase in urea excretion resulted. When the free bases were fed to human beings, there was an apparent conversion to other products, since the free bases did not appear in the urine. Free thymine was excreted when thymus nucleic acid was administered to a dog. Deuel concluded that normally the pyrimidine bases are not present in sufficient quantity to be excreted in the free unchanged state.

The failure to note metabolism of the free pyrimidine bases by some

of these early workers may have been due to the large amounts of pyrimidine bases administered; the small amount of pyrimidine catabolized may have easily escaped detection by the insensitive analytical methods employed. The methods of pyrimidine analysis in use before the advent of chromatography and spectrophotometry were hardly sensitive enough to make an accurate pyrimidine balance study possible.

Cerecedo (1927) reported that uracil and thymine were metabolized by dogs to yield urea. This was a confirmation of the work of Steudel (1910). Cytosine and 5-methyl cytosine were partly excreted unchanged, and partly deaminated to uracil and thymine, respectively. Hahn and Haarman (1926) had earlier reported that 5-methyl cytosine is deaminated and converted into thymine by autolyzates of bottom-fermenting brewer's yeast.

Emerson and Cerecedo (1930) found that uridine and cytidine, in contrast to the free base cytosine, were broken down to urea when fed to dogs in small amounts. Cerecedo (1930) reported that isobarbituric acid isodialuric acid were also metabolized to urea in dogs and he proposed the following scheme:

Uracil → isobarbituric acid → isodialuric acid →

urea + unknown carbon compound.

Cerecedo (1931a) reported later that oxaluric acid and formyloxaluric acid gave evidence of being partially catabolized to yield urea. He sug-

gested that oxaluric acid may be a connecting link in the catabolism of purines and pyrimidines. He also studied the fate of parabanic acid, alloxan and alloxantin. Parabanic acid (Cerecedo, 1931b) was found to be excreted to a great extent unchanged, with some conversion to oxalic acid. Alloxan was excreted as murexide in the urine and as an ethereal sulfate in the bile. Alloxantin was partially excreted in bile and was found in urine as murexide. The formation of formyloxaluric acid and oxaluric acid in the oxidation of uracil in vitro suggested that these two substances might also be intermediates in the catabolic breakdown of uracil in vivo (Cerecedo, 1933). The previously postulated reaction sequence was revised (Cerecedo, 1931a, 1933) to include oxaluric acid and oxalic acid:

Uracil → isobarbituric acid → isodialuric acid →

oxaluric acid → urea + oxalic acid.

Since thymine glycol when administered to dogs resulted in urea production, Cerecedo (1927) postulated that thymine is converted to thymine glycol:

Thymine → thymine glycol → urea.

The indirect nature of the evidence for these proposed reaction sequences leaves some doubt as to their actual occurrence.

Recent catabolism studies

Plentl and Schoenheimer (1944) studied the fate of N^{15}-labelled

pyrimidines when administered to rats and pigeons. These workers found no connection between the creatine cycle and purine-pyrimidine metabolism. The conversion of uracil and thymine to urea and ammonia observed by Cerecedo was confirmed. They concluded that pyrimidines are completely metabolized when administered in small amounts.

Bendich, et al. (1949) reported the synthesis of isotopic cytosine labeled with N^{15} in the 1 and 3 positions. When this compound was administered to the rat, isotopic nitrogen in urea and in ammonia resulted. This showed that at least some complete degradation of cytosine occured, and these investigators also concluded that some direct conversion to urea had taken place. Cytosine was catabolized in much smaller amounts than was the case with uracil and thymine in the experiments of Plentl and Schoenheimer (1944).

Hahn and Haarman (1926) observed that brewer's yeast autolyzates converted cytosine to uracil. Chargaff and Kream (1948) found that cell-free extracts of yeast converted cytosine to uracil through the action of cytosine deaminase. The enzymatic reaction was demonstrated in Escherichia coli (E. coli) and in the yeast with paper chromatography, by carrying out the reaction directly on the paper. Either the bacterial cells and substrate, or the cell-free yeast extracts and substrate were applied.

Hitchings, et al. (1948) reported that the growth of Lactobacillus casei in the presence of folic acid was inhibited by 2,4-diaminopyrimidine.

Bendich, et al. (1950) synthesized this compound with an excess of N^{15} in positions 1 and 3 as well as in the 2-amino group. The compound was administered to the rat. These investigators found that 2,4-diamino-pyrimidine was extensively absorbed by the rat, but the data indicated that it was not metabolized to ammonia, urea, or allantoin, nor was it utilized for the biosynthesis of nucleic acids.

Di Carlo, et al. (1951) reported that Saccharomyces cerevisiae showed limited growth when adenine, guanine, and cytosine were added to the medium as sole sources of nitrogen. Torulopsis utilis extensively utilized all of the naturally occurring purines and pyrimidines tested as sole sources of nitrogen, with the exception of thymine and alloxan. Pyrimidine nucleosides and nucleotides as well as other substances tested did not serve as nitrogen sources. Thymine and 2-aminopyrimidine were not antagonistic to uracil utilization. Di Carlo, et al. (1952a) studied diazobarbituric anhydride, in conjunction with all the purines, pyrimidines, and related compounds as sole sources of nitrogen for T. utilis and S. cerevisiae. Apparently it was not metabolized, but served to antagonize the assimilation of cytosine and 5-methyl cytosine by S. cerevisiae. This observation was interpreted to indicate that cytosine deaminase was inhibited by this compound.

Di Carlo, et al. (1952b) reported that T. utilis assimilated all of the nitrogen in the uracil molecule for growth, but failed to grow on

isobarbituric or isodialuric acid. This showed that the yeast did not catabolize uracil by the pathway which Cerecedo postulated to occur in dogs.

Hayaishi and Kornberg (1952) studied pyrimidine metabolism in whole cells and partially purified enzymes of species of Corynebacterium and Mycobacterium isolated from soil. They suggested that these pathways occur in these organisms:

Cytosine → uracil → barbituric acid → urea + malonic acid

$$\rightarrow 2NH_3 + CO_2$$

5-methyl cytosine → thymine → 5-methyl barbituric acid →

X → $CO_2 + H_2O + NH_3$.

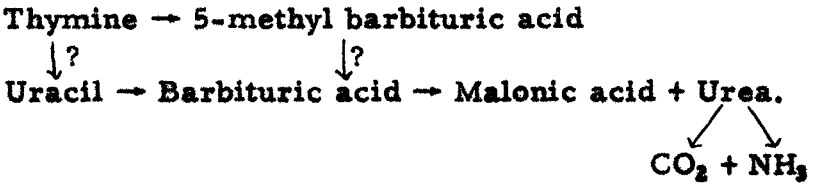
The oxidation of uracil and thymine at carbon 6 to yield the barbituric acids was shown to be due to the action of a single enzyme, "uracil-thymine oxidase". An enzyme "barbiturase" which catalyzed the hydrolysis of barbituric acid to urea and malonic acid was partially purified and freed of urease. The oxidation of 5-methyl barbituric acid has been observed only with intact cells and the mechanism has not been established.

Wang and Lampen (1952a and 1952b) found that a soil bacterium utilized uracil, cytosine, thymine or barbituric acid as sole sources of nitrogen. Data with resting cells indicated that the 6-position of the ring is the initial position involved in oxidation reactions. Only 60-70 % of the C and N following oxidation of the three pyrimidines cytosine, uracil, and

thymine were recovered as CO_2 and NH_3. Some evidence for assimilation was obtained from experiments with sodium azide. Orotic acid gave no oxygen uptake, but could be utilized slightly for growth. All 5-substituted uracil or cytosine compounds were readily oxidized by this organism.

Wang and Lampen (1952b) later reported that cell-free extracts of this soil bacterium contained the enzyme uracil oxidase, but not cytosine deaminase. Barbituric acid was isolated from the reaction mixture following uracil oxidation.

Lara (1952a and 1952b) has demonstrated the utilization of thymine or uracil as the sole source of energy, carbon, and nitrogen for several species of Corynebacterium and Nocardia. In all cases, it was found that the degradation of pyrimidines was brought about by adaptive enzymes. The results indicated that the pathways proposed by Cerecedo do not apply to Nocardia corallina. Cell-free enzyme preparations of this organism showed activity against thymine and uracil only when methylene blue was added to the reaction mixture. He concluded that oxidation of these two pyrimidines resulted in 5-methyl barbituric acid and barbituric acid, respectively. Barbituric acid was shown to be hydrolyzed by the enzyme preparation with the formation of one mole of carbon dioxide, two moles of NH_3 and one mole of malonic acid per mole of substrate used. Lara suggested the following reaction sequence:



Thymine resulted in uracil formation in one experiment, but the failure of this reaction to occur in many other experiments led to the conclusion that this does not normally occur.

Lieberman and Kornberg (1953a, 1953b) suggested that the metabolism of orotic acid by cell-free preparations from an anaerobic bacterium proceeds as follows:

I Orotic acid + $DPNH_2$ → Dihydroorotic acid + DPN

II Dihydroorotic acid + H_2O → Ureidosuccinic acid

III Ureidosuccinic acid → 5-acetic acid hydantoin.

These workers report that synthetic ureidosuccinic acid is converted to orotic acid at a rapid rate when coupled with $DPNH_2$ oxidase. These investigators have not as yet determined whether 5-acetic acid hydantoin lies on the main pathway of orotic acid metabolism. The enzyme which catalyzes reaction (1) has been partially purified. Dihydroorotic acid has been isolated in crystalline form, and is as effective as orotic acid for the growth of Lactobacillus bulgaricus 09. This organism utilizes orotic acid and ureidosuccinic acid for the synthesis of nucleic acid pyrimidines. The equilibrium strongly favors orotic acid reduction.

Pyrimidine Biosynthesis

Rat studies

Barnes and Schoenheimer (1943) reported that NH_3 is a source of pyrimidine nitrogen in the rat. Reichard (1952) using $N^{15}H_4Cl$ showed that the isotopic nitrogen from this source is incorporated into orotic acid in the rat.

Heinrich and Wilson (1950) studied the fate of C^{14}-labeled bicarbonate, acetate, glycine, and formate, administered to rats. Their experiments showed that CO_2 is the precursor of the ureide carbon of uracil and thymine in rats. Formate, glycine, and acetate were not incorporated into the pyrimidines of the rat nucleic acid. While the formate carbon serves as the precursor of carbon 2 in the purine ring, in the presence of folic acid, this vitamin is not similarly concerned in pyrimidine synthesis (Wright, 1951).

Lagerkvist (1950) in similar experiments with rats also showed that carbon atom 2 of uracil is derived from CO_2. Le Page and Heidelberger (1951) failed to find glycine-2-C^{14} incorporated into the pyrimidines of rats. This observation confirms the results of earlier experiments by Heinrich and Wilson (1950).

Elwyn and Sprinson (1950) in experiments with C^{14}-labeled serine and glycine found that the beta carbon of serine and the alpha carbon of

glycine served as sources of the methyl group of thymine in the rat. They interpreted their data to indicate that the methyl group is not introduced together with carbon 5 of the pyrimidine ring. The probability that a single carbon intermediate serves as the source of the methyl group presented itself.

Totter, et al. (1951) using radioactive formate labeled with C^{14} found that its carbon was incorporated into the 5-methyl group of thymine in rat desoxyribonucleic acid. These works postulated a conversion of formate to serine. The data indicated that although formate is directly assimilated into the purines, the activities of cytosine and uracil may be derived from formate via a more circuitous route, probably through carbon dioxide.

Goldthwait and Bendich (1952) confirmed this work. Using C^{14} - labeled formate administered to rats, they also found incorporation of radiocarbon in the thymine portion of the nucleic acids.

Elwyn, et al. (1951) studied the fate of 2, 3 deuterio-3-C^{14}-N^{15} serine. On the basis of these studies, he questioned whether formate is necessarily the intermediate in the transfer of the beta carbon of serine to the methyl group of thymine. The data indicated that both beta hydrogen atoms accompanied the carbon in the conversion of the serine beta carbon to the methyl groups of choline and thymine. The beta carbon of serine did not appear to go through formate, because that would result in the loss of at

least half of its deuterium. This conclusion is based on the assumption that there would be an equal amount of unlabeled hydrogen atoms and labeled hydrogen atoms being removed from the beta carbon.

Hammersten and co-workers (Bergström, et al., 1949; Arvidson, et al., 1949) first showed that N^{15}-labeled orotic acid was incorporated into the pyrimidine fractions of rat nucleic acids. Weed, et al., (1950) showed incorporation of C^{14}-labeled orotic acid-4-C^{14} into the pyrimidine nucleotide fractions of rat liver slices in vitro. Similar incorporation of labeled orotic acid was found in rat and human tumors (Weed, 1951).

Lagerkvist, et al. (1951) tested labeled aspartic acid using slices of regenerating rat liver. They found that the methylene carbon (and less extensively, the gamma-carboxyl carbon) of aspartic acid were utilized for pyrimidine synthesis. Labeled aspartate (C^{13}, C^{14}), N^{15}-labeled aspartate, and labeled pyruvate (C^{13}, C^{14}) were tested, but only the first substance showed activity in the uracil recovered. These observations indicated that the NH_2 group is lost, after which the remainder of the aspartic acid molecule is probably utilized for uracil synthesis via a relatively direct route. The labeled amino group did not appear in the uracil fraction.

Bergström, et al. (1951) reported that N^{15}-labeled 5-acetylhydantoin and 5-carboxymethylidenehydantoin were not incorporated into the nucleic acid of rat liver. These compounds were mentioned as chemical inter-

mediates in the orotic acid synthesis described by Nye and Mitchell (1947).

Studies with microorganisms

Molds. Mitchell and co-workers have studied pyrimidine biosynthesis through the use of various Neurospora crassa mutants. Mitchell and Houlahan (1947) reported that one of three genetic types of pyrimidineless mutants can utilize orotic acid to replace the requirement for uracil. They suggested, however, that probably neither pyrimidine is a normal precursor of uridine. They presented data to indicate that the carbon chain of pyrimidines arises from oxalacetic acid. They suggested that uridine is formed biologically through formation of intermediate aliphatic derivatives of ribose.

Houlahan and Mitchell (1947) investigated certain mutants of Neurospora which appeared to contain a "suppressing" agent. This suppressor acted upon three physiologically different pyrimidine-requiring strains which behaved in genetic tests as though they carried allelic genes. The requirement of these mutants for uridine or hydrolyzed nucleic acid were much the same if grown at 35° C., but at 25° C., there were striking differences. At 25° C., mutants 3b and 3c almost lost their requirement for the hydrolyzed nucleic acid. Houlahan and Mitchell (1948) later reported evidence for an interrelation in the metabolism of lysine, arginine, and pyrimidines in Neurospora. Pyrimidine mutant 3a has an absolute

requirement for pyrimidine, while 3a-s (where s = a mutant gene which represses the effect of 3a) again requires pyrimidine if arginine is added to the medium. Citrulline and ornithine were found to be somewhat less inhibitory than arginine. The inhibition by arginine could be overcome by hydrolyzed nucleic acid or lysine. The authors suggested that perhaps one or both amino acids were involved in pyrimidine biosynthesis in this organism. Two lysineless mutants were found to accumulate pyrimidines, two of which were identified as uridine and uracil.

Loring and Pierce (1944) reported that orotic acid could be substituted for uracil in satisfying the growth requirements of some pyrimidine-requiring mutants of Neurospora crassa.

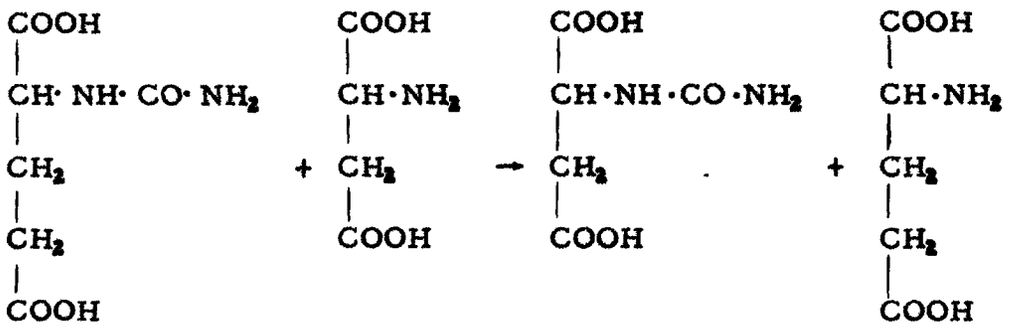
Mitchell, et al. (1948) reported that three mutants of Neurospora accumulated large amounts of orotic acid during growth in the presence of cytidine. These observations were interpreted to indicate that orotic acid has a significant place in nucleic acid biosynthesis.

Bacteria. Chattaway (1944) and Rogers (1944) found that orotic acid had growth promoting activity for Lactobacillus casei E and Streptococcus hemolyticus C7, respectively.

Wright and co-workers (Wright, Huff, et al., 1950; Huff, et al., 1950) and Wieland, et al. (1950), working independently, reported that Lactobacillus bulgaricus 09 required orotic acid for growth. Wright, et al. (1951) showed that this organism converted either orotic acid-2-C^{14} or ureidosuccinic

acid-ureido-C^{14} into both the uracil and cytosine of the polynucleotides. Later work by Wright and Miller (1952) showed that the organism could utilize uracil instead of orotic acid provided the incubation period was greatly prolonged.

Wright (1951) proposed a mechanism for ureidosuccinic acid formation whereby aspartic acid is converted by transcarbamylation with ureidoglutaric acid to yield ureidosuccinic acid + glutamic acid:



Ureidoglutaric acid — Aspartic acid — Ureidosuccinic acid — Glutamic acid

If the mechanism suggested by Wright occurs, then it is reasonable to suppose that biotin may be essential to pyrimidine synthesis, since this vitamin appears to be concerned with synthesis of alpha-ureidoglutaric acid (Feldott, et. al., 1950).

Weed and Cohen (1951) found that labeled orotic acid was incorporated into the pyrimidines of Escherichia coli "B" which in turn were utilized in the synthesis of T_6r^+ and T_6r viruses.

Back and Woods (1953) reported that a strain of E. coli (B. coli) which required uracil would also grow on ureidosuccinic acid although the lag

phase when grown in the presence of the latter compound was increased 7 to 20 hours. Growth on ureidosuccinic acid was inhibited by the higher homolog, ureidoglutaric acid, when the ratio of ureidoglutarate to ureidosuccinate was 15 to 1 or greater, and the range of ureidosuccinate concentration was 10^{-4} to 5×10^{-3} M. Growth on uracil was not affected by even the highest concentration of ureidoglutarate. These observations are difficult to reconcile with the proposed mechanism of Wright (1951). It would be expected that the addition of ureidoglutarate would increase ureidosuccinate formation if the mechanism proposed by Wright (1951) occurs. However, since the structure of ureidoglutaric acid is similar to ureidosuccinic acid itself, it could act as a competitive inhibitor at relatively high concentrations. This could occur even though it does increase the actual formation of ureidosuccinic acid, through transcarbamylation.

Yeasts. Pomper (1952) reported that two pyrimidineless mutants of Saccharomyces cerevisiae utilized uracil, cytosine, 5-nitrouracil, uridine, or uridylic acid. Cytosine and uracil were utilized at least as efficiently and rapidly as the corresponding nucleosides and nucleotides.

Edmonds, et al. (1950, 1952) studied purine and pyrimidine metabolism in Torulopsis utilis. Growth of the yeast in the presence of C^{14}-labeled formate and C^{14}-methylene labeled glycine resulted in incorporation of formate and methylene carbon into guanine and adenine of the yeast nucleic acid, but not into uracil. Sodium lactate-2, 3-C^{14} was incorporated

into the guanine and uracil of the nucleic acid, while orotic acid-6-C^{14} was incorporated into uracil only. These investigators suggested that lactate is converted to oxalacetate prior to its incorporation into the uracil molecule.

Biosynthesis of Nucleosides and Nucleotides

Utilization of pyrimidine bases

Snell and Mitchell (1941) reported various purine and pyrimidine bases as growth substances for lactic acid bacteria. They found uracil greatly stimulatory to the growth of Lactobacillus arabinosus, and somewhat stimulatory to L. mesenteroides. Thymine was found to be essential to the growth of Streptococcus lactis. In general, these workers found that the naturally occurring amino derivatives of the purine or pyrimidine bases are replaceable by the corresponding oxy-derivatives. Thus cytosine and uracil are interchangeable as well as guanine and xanthine.

Plentl and Schoenheimer (1944) found that the free bases uracil and thymine are not utilized in rats and pigeons for synthesis of nucleoproteins. They felt that they must either be supplied in the form of nucleosides, nucleotides, or nucleic acids, or else they are synthesized from smaller molecules. The failure of these organisms to utilize free pyrimidine bases was difficult to explain, since in all their other metabolic investigations, the addition to the diet of substances which are normal tissue constituents led to their deposition as an inseparable mixture of the preformed and dietary material.

Bendich, et al. (1949) administered N^{15}-labeled cytosine to rats, but found no incorporation of the labeled nitrogen in the nucleic acid fraction

of the tissues.

Moore and Boylen (1953) reported that a pyrimidine-requiring strain of Escherichia coli utilized uracil as the sole source of the pyrimidine portion of its ribonucleic acid. Uracil-2-C^{14} was added to the growth medium. The cytidylic acid and uridylic acid resulting from hydrolysis of the ribonucleic acid of the cells showed activity of the same magnitude as that exhibited by the original labeled uracil. These results pointed to a high incorporation of the free base, in contrast with the results of Plentl and Schoenheimer (1944) with rats and pigeons.

Riboside and ribotide utilization

Loring and Pierce (1944) observed that the pyrimidine nucleosides and nucleotides were from 10-60% more effective for the growth of a strain of Neurospora crassa than the free bases alone.

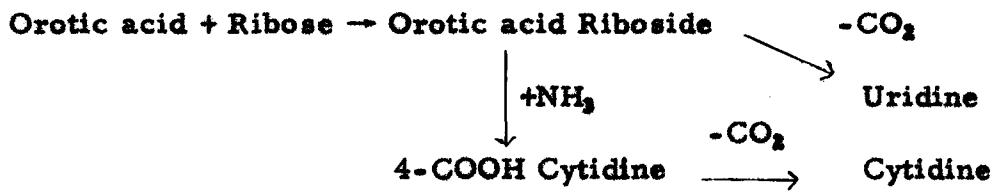
Merrifield and Dunn (1950) found that cytidine and uridine gave 90% of the response elicted by the free pyrimidines with Lactobacillus brevis, while the effect of the corresponding nucleotides was about equal to that of the free bases. Orotic acid could completely satisfy the pyrimidine requirement of this organism. However, the uracil requirement of L. helveticus could not be satisfied by orotic acid, cytidine, uridine, cytidylic acid or uridylic acid.

Hammersten, et al. (1949) and Hammersten, et al. (1950) studied the

utilization of pyrimidine ribosides and desoxyribosides for rat nucleic acid synthesis. They used labeled cytidine and uridine which had been isolated from yeast grown in the presence of N^{15}-ammonia. The injected cytidine-N^{15} entered the RNA in the low dilution while the uridine was incorporated to a much lesser degree. The injected cytidine appeared not only to be a precursor of the cytidine of the RNA but also for the uridine. The results indicated that cytidine is the precursor of both RNA and DNA pyrimidines. In view of earlier work (Bergstrom, et al., 1949; Arvidson, et al., 1949) which had shown the incorporation of orotic acid into rat nucleic acid pyrimidines, these investigators reasoned that perhaps orotic acid may first be transformed to cytidine and that uridine is formed from this cytidine. Reichard's experiments (1949) however, had led to the conclusion that uridine and cytidine are formed from orotic acid independently of one another.

Loring and Pierce (1944) suggested that the first step in the transfer of orotic acid to cytidine and uridine might be the decarboxylation of orotic acid riboside to uridine.

Arvidson, et al. (1949) proposed the following scheme:



Reichard and Estborn (1951) isolated N^{15}-labeled pyrimidine desoxy-

ribosides from Escherichia coli cells which had grown in a medium containing $N^{15}H_4^+$ ions. These labeled desoxyribosides were incubated with non-growing and regenerating rat liver. The results showed that both cytidine and thymine desoxyribosides were utilized by rat liver for replacement of DNA pyrimidines. There was no incorporation into the RNA fraction. Regenerating rat liver showed at least three times the incorporation of pyrimidines observed by non-growing rat liver.

Pierce and Loring (1948) reported purine and pyrimidine antagonism in a pyrimidine-deficient mutant of Neurospora. Utilization of the pyrimidine ribonucleosides and nucleotides in a pyrimidine-deficient mutant could be completely inhibited by the addition of adenosine or adenosine-3-phosphate. Adenine had no antagonistic effect in comparable concentrations. The nucleotides were more readily inhibited than the nucleosides, and cytidylic acid and cytidine were more easily inhibited than uridylic acid or uridine. Guanosine and guanylic acid also produced an inhibitory effect, but larger amounts were required. Inhibition of cytidine by adenosine was reversed by an amount of uridine equal to that of the cytidine. Uridine inhibition by adenosine was not removable by the addition of a comparable amount of cytidine, however. These observations suggested that the deamination of cytidine to uridine as well as the utilization of uridine were inhibited by the purine compounds.

Riboside accumulation

Michelson, et al. (1951) isolated a new pyrimidine riboside from a mutant strain of Neurospora. The substance accumulates when the organism is grown on a cytidine-containing medium. It has a characteristic absorption spectrum. In contrast to the common pyrimidine ribosides, it is readily hydrolyzed, and chemical tests have shown that the components are orotic acid and ribose. Accordingly, these investigators have designated the compound orotidine. Orotidine had been postulated by several investigators as a probable intermediate in pyrimidine metabolism (Loring and Pierce, 1944; Arvidson, et al., 1949; Schlenk, 1949; Paege and Schlenk, 1950b).

Nucleoside metabolism

Paege and Schlenk (1950a, 1950b) have investigated the metabolism of pyrimidine ribosides. They reported that cytosine riboside and uracil riboside were rapidly metabolized by bacterial enzymes. Cytidine was first deaminated; the pyrimidine base was next split off through the action of nucleoside phosphorylase. Uracil was accumulated, while the carbohydrate component disintegrated rapidly after the splitting off of the pyrimidine base. These observations were similar to those described earlier for purine ribosides (Schlenk, 1949). No indication was found that the action of bacterial pyrimidine nucleoside phosphorylase was

preceded by opening of the pyrimidine ring as suggested by Mitchell and Houlahan for Neurospora (1947). Two alternative schemes were suggested as possible reaction sequences undergone by orotic acid:

1. Orotic acid $\xrightarrow{-CO_2}$ Uracil → Uridine
2. Orotic acid $\xrightarrow{-CO_2}$ Orotic acid Riboside $\xrightarrow{-CO_2}$ Uridine

A pyrimidine nucleosidase from Escherichia coli was purified by these workers which showed specificity toward uridine and catalyzed the reversible splitting of this nucleoside by phosphorolysis (Paege and Schlenk, 1952). Ribose-1-phosphate was accumulated.

Wang, et al. (1950) and Lampen and Wang (1952) have studied the enzymatic deamination of cytosine nucleosides. Extracts of E. coli were prepared which deaminated cytidine and cytosine desoxyriboside. These extracts deaminated the desoxyriboside three to four times more rapidly than the riboside. Indications were that it is a hydrolytic deamination; one mole of ammonia was formed per mole of nucleoside.

Wang and Lampen (1952b) found that cell-free extracts of a soil bacterium contained a pyrimidine nucleoside phosphorylase which acted more rapidly on thymidine than on uridine or cytidine.

Friedkin and Roberts (1951) found that desoxyribose-1-phosphate and thymine were converted to thymidine in the presence of a fractionated enzyme preparation obtained from calf kidney.

McNutt (1952) reported that certain enzyme preparations from

Lactobacillus helveticus S were able to catalyze the transfer of the desoxyribosyl group from one purine or pyrimidine to another.

Dinucleotide utilization

Merrifield and Woolley (1952) have isolated several dinucleotides from acid-treated yeast ribonucleic acid. These dinucleotides and their dephosphorylated derivatives were tested for their ability to replace the uracil requirement of Lactobacillus helveticus 335. The smallest microbiologically active unit was cytidine-5'-phosphate diesterified at the 3'- (or 2'-) position of another nucleoside. All the active compounds contained this unit, while those which did not were inactive. It was not ascertained whether uridine could replace cytidine in this unit. These investigators interpreted their results to indicate that L. helveticus 335 utilized uracil not by conversion to a nucleoside or mononucleotide, but by direct incorporation into a dinucleotide.

Recent Nucleotide Isolations

Cytidine nucleotides

Loring and co-workers (Loring and Luthy, 1951; Loring, et al., 1952a, 1952b) have isolated two isomeric cytidylic acids from yeast ribonucleic acid. The physical and chemical properties of these compounds have been reported in detail. Several lines of evidence were presented to

show that dephosphorylation of the two acids leads to the formation of the same cytidine. The results indicate that the isomerism must involve the location of the phosphate group in the 2' position in one case and in the 3' position in the other. The two cytidylic acids have been isolated in crystalline form.

Uridine nucleotides

Caputto, et al. (1950) reported the isolation of uridine-5'-diphosphate glucose from baker's yeast which serves as the coenzyme for galactowaldenase. Galactowaldenase catalyzes the conversion of galactose-1-PO_4 to glucose-1-PO_4.

Cabib, et al. (1953) have isolated uridine diphosphate acetylglucosamine from baker's yeast.

Hurlbert and Potter (1952), and Hurlbert (1953) injected orotic acid-6-C^{14} into rats and the localization of the C^{14} was determined in the nucleic acids and the perchloric acid extracts of the liver. Most of the C^{14} in the extracts was found in uridine-5'-phosphate or its derivatives. The specific activity of the uridylic acid from the nuclear ribonucleic acid was about one-half that of the uridine-5'-phosphate. These investigators concluded that uridine-5'-phosphate compounds occupy an important place in nucleic acid metabolism.

Dutton and Story (1953) reported the isolation of a compound of

uridine diphosphate and glucuronic acid from liver. This compound is necessary for the formation of o-aminophenyl glucuronide from o-aminophenol in liver homogenates.

Buchanan, et al. (1953) have identified two nucleotide coenzymes which are implicated in photosynthesis, uridine-5'-diphosphate glucose and uridine-5'-diphosphate galactose.

Park and Johnson (1949) found that in the presence of penicillin Staphylococcus aureus H accumulated three compounds which contained uridine-5'-phosphate. Park (1952a, 1952b, 1952c) reported that the compounds also contained an amino sugar which was linked to the secondary phosphoric acid group by an acetal-like bond. The amino sugar is unusual in that it appears to be an N-acetyl-2-amino sugar which possesses a carboxyl group. All three compounds have been isolated. One compound consists of uridine-5'-pyrophosphate attached to the amino sugar. The second compound is similar, but in addition contains L-alanine which probably is attached to the carboxyl group of the amino sugar. The third compound contains a peptide which is attached to the substituted sugar. The peptide is composed of L-lysine, D-glutamic acid and three alanine residues. These three compounds are apparently synthesized by the growing cells only during the short period they remain viable after the addition of penicillin. The significance of these compounds in the economy of the cell is not clear.

Uridine triphosphate was first isolated from yeast in the laboratories of Pabst Brewing Company, Milwaukee, Wisconsin (1953).

These recent studies indicate that the uridine-5'-phosphates are an important class of compounds. In many ways they are similar to the adenine compounds, AMP, ADP, and ATP. Their role in energy transfer has been implicated (Kornberg, 1951).

MATERIALS AND METHODS

Organisms

The organisms utilized in this study were Aerobacter aerogenes NRRL #199 and mutant strains of this parent culture. The cultures were maintained on nutrient agar slants and transferred every three months. All cultures were incubated at 30° C.

Culture Media

The "complete" medium consisted of 0.5% tryptone, 0.3% yeast extract, 0.5% glucose, 0.3% $K_2HPO_4 \cdot 3H_2O$ and 0.1% KH_2PO_4, made to volume with distilled water.

The peptonized milk medium used was composed of 1.5% peptonized milk and 0.75% $K_2HPO_4 \cdot 3H_2O$, made to volume with distilled water. The phosphate was dissolved in water and the pH was adjusted to neutrality with concentrated H_3PO_4. This solution was sterilized separately and added aseptically to the remainder of the medium prior to inoculation. When prepared in 4.5 to 9 liter amounts, the solutions were sterilized by autoclaving at 15 pounds pressure for 25 minutes.

The minimal medium described by Davis and Mingioli (1950) was used. This medium contained 0.1% glucose, 0.7% $K_2HPO_4 \cdot 3H_2O$, 0.3% KH_2PO_4, 0.05% sodium citrate pentahydrate, 0.01% $MgSO_4 \cdot 7H_2O$,

and 0.1% $(NH_4)_2SO_4$. Distilled water was added to volume. When a solid minimal medium was desired, 1.5% agar was added to the medium. In making an agar medium, the glucose and agar were autoclaved separately from the remaining components. This precaution was taken to avoid caramelization of the glucose, which occurs when this sugar is autoclaved in the presence of phosphate. This practice also served to prevent the formation of colloidal agar particles, which so often occurs when phosphate and agar are autoclaved together.

The nutrient agar medium had the following ingredients: 0.5% peptone, 0.3% beef extract, 0.5% NaCl, 1.5% agar and distilled water to volume.

Production and Isolation of Mutants

The mutants were produced and isolated according to the penicillin technique (Davis, 1948; Lederberg and Zinder, 1948). The parent organism, Aerobacter aerogenes # 199 was transferred from a nutrient agar slant to a tube of "complete" broth. After incubation for four hours, the culture was transferred to another tube of "complete" broth and again incubated four hours. The culture was then centrifuged, washed with physiological saline, centrifuged again, and suspended in saline. About 2 ml of this bacterial suspension was transferred aseptically to a sterile Petri plate and irradiated for ten seconds with ultraviolet light emitted by

a Hanovia lamp at a distance of 15 cm from the light source. This treatment resulted in a killing of over 99.99% of the cells. (A very high killing effect is desired, in order to insure that the survivors will consist of a large percentage of mutants.) The irradiation was carried out with the cover of the Petri plate removed and the plate containing the suspension was rotated constantly during the irradiation period. After the exposure to ultraviolet light, 0.2 ml. of the suspension was transferred to a tube of "complete" broth. After incubation for 18 hours, 0.1 ml. of the broth culture was transferred to another tube of "complete" broth. After incubation for four hours, the culture was centrifuged. The cells were washed and finally suspended in saline. This suspension was used to inoculate tubes containing 10 ml. of minimal medium.

The suspension was added to the tubes dropwise until a faint turbidity was produced. A saline suspension of penicillin was then added in an amount sufficient to produce a final concentration of 300-400 units per ml. A level of 350 units per ml. usually proved most successful.

It is known that penicillin acts only on actively growing cells. For this reason, the minimal medium was routinely supplemented with amino acids, in order to remove most of the amino acid mutants as well as wild type cells. In order to select for dinucleotide mutants or mutants blocked still higher up in the reaction sequence, the free pyrimidine bases and their nucleosides and nucleotides were also added to the minimal medium.

Here again, the penicillin would destroy the unwanted mutants which grew in the supplemented medium.

After 5-8 hours incubation (when lysis could be observed) the culture was serially diluted and inoculated on nutrient agar plates. The inoculations were made by placing a drop of the suspensions on the nutrient agar surface, and spreading this drop evenly over the agar by means of a sterile glass spreader.

These plates were incubated for 24 hours and inspected for colony growth. Plates containing 25-100 colonies were selected for transfer to minimal agar and to minimal agar containing various supplements.

The replicate plating method (Lederberg and Lederberg, 1952) was used to transfer the colonies from the nutrient agar plates. Small pieces of sterile cotton velvet, 7 inches square, were placed over a cylinder 3 1/4 inches in diameter. The velvet squares were stretched taut and fastened to the cylinder with a rubber band. A nutrient agar plate containing colonies of organisms to be tested was inverted, with moderate pressure, onto the velvet so that the velvet retained a small part of each colony. The plates containing plain minimal medium and minimal-supplemented media were then successively inverted onto the velvet with slight pressure in order to effect an inoculation. A nutrient agar plate was inoculated last. After incubation for 24 hours, the mutant colonies were detected by matching the minimal plate with the nutrient agar control plate. The colonies which appeared on the nutrient agar plate but

did not grow on the minimal agar plate were assumed to be mutants. Examination of the other plates revealed whether they had responded to any of the supplements used. These colonies were transferred to nutrient agar slants, incubated, and kept for further study. They were retested by inoculating into liquid minimal medium which contained the various supplements, and identified according to their growth responses.

The replica plating method is very time-saving but is not infallible. One major defect is the tendency for too large an inoculum carry-over. In a large inoculum, enough essential metabolites may be present to enable mutants to grow even on a minimal agar medium.

Occasionally, therefore, this method was supplemented by liquid medium studies. Saline suspensions were made of the colonies to be tested, and inoculations were made from these suspensions into tubes of sterile minimal media to which various supplements had been added. The tubes were incubated for 18 hours and growth was measured turbidimetrically. Readings taken after 20-24 hours were found to be undependable due to a high reversion rate.

Double mutants were produced by irradiating mutants with single growth requirements. The same process of isolation and identification described for single mutants was used, except that in these studies, the minimal and supplemented media contained the compound required for growth by the single mutant.

Recombination Experiments

The technique employed in the recombination studies was essentially that of Lederberg (1947) and Tatum and Lederberg (1947). Mutants possessing double nutritional requirements were used.

Although it has been found that most single biochemical mutants revert spontaneously to prototrophs at the low rate of approximately 1 cell in 10^7, double mutants are even more stable. The probability of spontaneuos reversion occurring at both loci of a double mutant is $(10^7)^2$ or 1 cell in 10^{14}. In recombination studies it is highly important that stable mutants be used so that the appearance of growth can be ascribed with certainty to recombination and resultant production of nutritionally independent progeny. In view of these considerations, double mutants are more desirable for use in recombination studies than single mutants.

The mutants to be tested were grown separately in the "complete" medium. After incubation for four hours, the cells were centrifuged and washed twice in saline. Saline suspensions of the mutants were prepared containing 10^9 cells per ml. The mutants were incubated in pairs in all possible combinations by adding one-tenth ml. of a cell suspension of one mutant to one-tenth ml. of a cell suspension of another mutant. One-tenth ml. of the cell suspension of each mutant was also incubated singly, as a control. The single and mixed suspensions were incubated for various periods of time and then inoculated into solid or liquid minimal

media.

The liquid minimal medium was contained in tubes in 10 ml. amounts. When a solid minimal medium was employed, the cells were added to 10 ml. of 1.5% minimal agar and mixed. The agar was then poured into a Petri plate containing a base layer of 10 ml. minimal agar. Semi-solid agar (0.7%) was used instead of 1.5% minimal agar in some experiments.

Synergism Experiments

Synergism was demonstrated by inoculating plates which contained 15 ml. of minimal agar supplemented with 1 micromole per ml. orotic acid, ureidosuccinic acid, or aspartic acid. Inoculations were made by streaking the plates with a loopful of a saline suspension of washed cells. The streaks of the organisms to be tested were made parallel to one another, approximately 1 inch apart. The plates were incubated 20-24 hours and then examined for any evidences of enhanced growth on the side of a streak adjacent to another streak.

Chemicals

The pyrimidine bases, nucleosides, nucleotides and amino acids used in this investigation were obtained from commercial sources. The orcinol used was a commercial brand, recrystallized from chloroform to remove impurities which reduce the final color intensity of the Mejbaum

pentose test. The copper phosphate used was prepared from a commercial source of copper carbonate, by adding phosphoric acid and drying. Ureidosuccinic acid was prepared according to the method of Nye and Mitchell (1947). A mixture of 13 grams aspartic acid, 8 grams of potassium cyanate, and 100 ml. of 1 N potassium hydroxide was allowed to stand at room temperature for 16 hours. The solution was acidified with concentrated hydrochloric acid, and after two hours of standing, the acid was filtered and recrystallized from water. The material melted at 178-180° C., the same range reported for ureidosuccinic acid by Nye and Mitchell. The oxalacetic acid and ureidoglutaric acid used had been prepared in this laboratory. The oxalacetic acid activity was determined according to the method of Krebs and Eggleston (1945). This method is based on the fact that Al^{+++} ions decarboxylate oxalacetic acid. The CO_2 evolved was measured manometrically using Warburg flasks with double side-arms. The solution in the main compartment contained the sample, and was 0.2 N with respect to HCl. The side-arms contained 1 ml. 0.75 M phthalate buffer and 1 ml. 33.3% aluminum sulfate solution. A bath temperature of 30.4° C. was used. After equilibration, the phthalate buffer and the aluminum sulfate were successively added. A control flask containing water was necessary in order to correct for the pressure changes which occurred when the different solutions were added and mixed. The CO_2 evolution was complete within 60 minutes. Recovery

experiments give yields approximately 5% below theory because of spontaneous decomposition of oxalacetic acid during equilibration.

The pyruvate and succinate used were commercial brands.

The minimal medium when supplemented with oxalacetate or pyruvate was sterilized by filtration through a sintered glass filter. All other supplements were added to the minimal medium and sterilized in the autoclave.

Preparation of Cells

When large quantities of cells were desired, 50 ml. of medium were initially inoculated and incubated for 4-6 hours. This culture was added aseptically to 500 ml. of medium and incubated 4-6 hours. The second culture was finally used to inoculate 4.5-9 liters of medium contained in a 12 liter Florence flask. The culture was aerated vigorously by passing compressed air through an aerating stone which was suspended in the flask. The culture was incubated with constant aeration for 24-30 hours. The cells were harvested in a Sharples centrifuge.

Resting Cell Experiments

The cells were grown on minimal medium and on minimal medium which contained 100 micrograms per ml. orotic acid, ureidosuccinic acid, and aspartic acid, respectively. The cultures were incubated with

constant aeration for various lengths of time. The cells were centrifuged and washed twice in sterile 0.07 M phosphate buffer before finally adding buffer in an amount sufficient to produce a final suspension consisting of from 1 to 5% cells by volume. The activity of the resting cells on various substrates was tested under aseptic conditions. The reaction mixture contained 0.07 M phosphate, M/36 glucose, 2 ml. of the cell suspension, and 1 micromole per ml. of substrate, in a total volume of 25 ml. The substrates tested were orotic acid, ureidosuccinic acid, and aspartic acid. The flasks were incubated on a shaker for 20-24 hours. The cells were then removed by centrifugation, and the supernatants analyzed for uracil. The supernatants from the flasks which contained ureidosuccinic acid or aspartic acid were analyzed for orotic acid as well as uracil.

Cell-free Extract Experiments

The cells were grown either on minimal media or on peptonized milk. After growth for 12 hours, with constant aeration, the cells were harvested in a Sharples centrifuge. The cells were washed with several liters of saline. The cells were resuspended in 100 ml. saline and homogenized in a Waring blendor. Then the suspension was again centrifuged in the Sharples centrifuge. Finally the cells were suspended in 0.130 M phosphate buffer, pH 7, using a cell-buffer ratio of 1:2 or 1:1 by weight. The cell-free extracts were prepared by subjecting 15-20 ml. of this

suspension to sonic disintegration for 30 minutes in a Raytheon 9-kc. Magnetostriction Oscillator. The cup contents were kept cold during the sonic vibrational treatment by circulating ice-cold water through the apparatus. The cell debris was removed immediately by centrifugation at 10,000 r.p.m. for 30 minutes in the high speed head of an International refrigerated centrifuge. The supernatant was removed and frozen immediately.

The extracts were analyzed for protein content by the Weichselbaum biuret test (1946). Usually 0.2 ml. enzyme preparation was used, and mixed with 2.5 ml. saline and 2.5 ml. Weichselbaum reagent. This mixture was allowed to stand for one hour at room temperature and then read in a Klett-Summerson colorimeter using a 540 mμ filter. The amount of albumin nitrogen represented by the Klett readings was ascertained by comparing the readings to a standard curve of known concentrations of albumin solutions. The extracts averaged about 3 mg. albumin nitrogen per ml. Attempts to increase the protein concentration of the extracts by changing the cell-buffer ratio of the suspension to be sonized from 1:2 to 1:1 were unsuccessful.

Warburg Studies

In experiments with the Warburg respirometer, the cell-free extracts were added to one side-arm in amounts varying from 0.2 ml. to

1 ml., and were added to the main flask after equilibration. The other side-arm contained 0.3 ml. H_2SO_4 which was added to the reaction mixture at the completion of the experiment in order to stop the reaction and to release all bound CO_2. The temperature of the bath was 30.4° C. in all experiments. A constant amount of phosphate buffer, pH 7, was added to all flasks. In oxygen uptake experiments, the CO_2 produced was absorbed by 0.2 ml. 40% NaOH in the center well of the Warburg flasks. A small piece of filter paper was placed in the center well to provide more surface area for CO_2 absorption by the NaOH. Anaerobic conditions were produced by passing nitrogen through the flasks after they were attached to the manometers, before placing the flasks in the bath. The nitrogen was freed of oxygen by allowing it to flow through a tube which contained red-hot copper wire. When the flask contents were analyzed for pyrimidines at the close of the experiment, protein was first removed by the addition of 1 ml. of 2.5 % trichloroacetic acid. The precipitate was removed by filtration and the filtrate used for chromatographic and spectrophotometric analyses.

Dubnoff Metabolic Shaker Experiments

A total reaction mixture volume of 2 ml. was used in experiments employing the Dubnoff shaker. Enzyme solutions were added in amounts varying from 0.2 ml. to 1 ml. Forty micromoles of substrate and a

constant volume of pH 7 phosphate buffer was added. The reaction mixtures were placed in small flasks in the bath and incubated 3-6 hours. The bath was maintained at 30° C. and the flasks were kept in constant motion. Samples were taken from the flasks at intervals and deproteinized with 2.5 % trichloroacetic acid. The mixture was heated slightly to facilitate precipitation of the protein, and the filtrate was analyzed chromatographically and spectrophotometrically for pyrimidines.

Paper Chromatography

The paper chromatograms were made with Whatman No. 1 filter paper which was cut into sheets 11 1/4 x 16 inches. The base line was drawn about 1 1/4 inches from the bottom of the sheet. The solutions were applied at 1 1/2 inch intervals along the base line, never less than 1 1/4 inch from the edge of the paper. Standard solutions were used for reference spots. Drying of the solutions placed on the paper was facilitated by a 110-120 volt, 60 cycle AC hair dryer, manufactured by the Superior Electric Products Corporation, Cape Girardeau, Missouri. Each chromatogram was rolled into a cylindrical shape upon completion of the applications, and fastened in three places with string so that the edges of the paper did not touch. The chromatogram was placed in a vertical position inside a glass cylinder 18 inches tall and 5 1/2 inches in diameter. Freshly prepared solvent was placed in the bottom of the

cylinder just prior to introducing the chromatogram. Enough solvent was used so that the paper was immersed to within 0.6 inch of the base line when placed in the glass cylinder. The volume of solvent used was usually 120 ml. For separation of the compounds under study, a solvent consisting of n-butanol, glacial acetic acid and water in a ratio of 4:1:1 by volume was employed. A solvent consisting of ethanol and water in a ratio of 4:1 according to volume was also used. The cylinder was made air-tight by pressing a piece of aluminum foil firmly over the top, the edges of which were coated lightly with Lubriseal. The chromatograms were allowed to stand in a dark room at 30° C. until the solvent front had reached the top of the paper or stopped moving near the top of the paper. After removal from the cylinder, the chromatograms were dried thoroughly and then viewed in a dark room under a Mineralight lamp Model SL 2537 with short wave ultraviolet filter. The pyrimidine and purine compounds appear as violet to purple spots against a flourescent background. If further study of these compounds was desired, the areas were delineated lightly with pencil, and cut out. The paper was cut in small pieces and eluted overnight in water or 0.1 N HCl. The filter paper was removed from the solution by filtration through a Medium sintered glass filter. The Beckman spectrophotometer was used for quantitative estimations of pyrimidines in the filtrates.

Spectrophotometric Studies

The Beckman spectrophotometer, model DU, was used to carry out qualitative and quantitative ultraviolet spectrophotometric measurements. The absorption curves were studied between 220-320 mμ.

Growth Studies

Growth was measured turbidimetrically, using a Klett-Summerson photoelectric colorimeter, with a 660 mμ filter, or an Evelyn colorimeter, with a 620 mμ filter.

Isolation and Identification of Unknown Metabolic Product

The unknown pyrimidine compound was isolated by the method described by Michelson, et al. (1951) for the isolation of orotidine from Neurospora. The harvested cells were suspended in water and 95 % alcohol in a ratio of 1:1:2 by volume. The resulting slurry was heated at 80° C. for 30 minutes and allowed to stand overnight. After centrifugation of this mixture, the residue was washed with 2 parts of hot 50 % ethanol. Barium acetate was added to the combined filtrates on a basis of 2.5 % of the original cell wet weight. The solution was adjusted to pH 8.5 with barium hydroxide. The resultant precipitate was removed by centrifugation, washed with distilled water, and recentrifuged. The solution resulting from the combined supernatants was evaporated under reduced

pressure to a small volume. The unknown compound was precipitated by the addition of a saturated solution of lead subacetate. The precipitate was centrifuged, washed with water, and suspended in a small volume of water. The solution was saturated with hydrogen sulfide, and the lead sulfide was removed by centrifugation. The filtrate from the lead sulfide precipitate contained the unknown compound. This solution was evaporated again to a small volume under reduced pressure, and isolated by paper chromatography. The area of the paper which contained the unknown compound was cut into small pieces and eluted overnight in 0.1 N HCl or water. The solution was filtered through a Medium sintered glass filter to remove filter paper particles. A filter paper control was cut out and treated similarly. The filtered solution was used directly for spectrophotometric study and chemical tests, along with the filter paper control.

Hydrolysis of Unknown Compound

The compound could be hydrolyzed by autoclaving in 8 N HCl at 15 pounds pressure, for two hours. This treatment produced sufficient hydrolysis so that uracil could be detected both chromatographically and spectrophotometrically.

Detection and Determination of Ribose

Ribose was determined qualitatively and quantitatively. Quantitative estimations were made by comparison with a standard curve prepared with known concentrations of uridine-3'-phosphate. The Paege (1951) modification of the Mejbaum test (1939) was used. This modification was especially adapted to the determination of pyrimidine-bound ribose. One ml. of the sample containing 1-15 micrograms pentose was used. To this was added 2.5 ml. concentrated HCl and 1.5 ml. orcinol reagent. This mixture was heated in a boiling water bath for 4 hours. After cooling, the mixture was readjusted to 5.0 ml. and read in a Klett-Summerson colorimeter with a 660 mμ filter. The 1 % orcinol reagent was prepared immediately before use by adding 15 mg. orcinol to 1.5 ml. of a 0.1 % $FeCl_3$ (in concentrated HCl) solution. Commercial orcinol products often contain impurities which interfere with this test, therefore the orcinol used was purified by recrystallization with chloroform.

Phosphate Determination

Phosphate was determined qualitatively and quantitatively using the method of Fiske and SubbaRow (1925). Total, stable, labile and inorganic phosphorus was determined by this method. The color was developed by mixing the reagents with a water solution of the sample containing 4-40 micrograms phosphorus. After standing for 3.5 minutes

the color intensity was read in a Klett-Summerson photoelectric colorimeter with a 660 mμ filter. Standards were prepared under identical conditions, using known amounts of KH_2PO_4. The amount of phosphorus was determined by comparing the reading of the unknown with the standard curve.

Total phosphorus was determined by ashing the sample. The sample was heated in a micro-Kjeldahl flask with 0.3 ml. of concentrated H_2SO_4 until white fumes of SO_3 appeared. Two or three drops of concentrated HNO_3 were next added, and heating was continued until brown fumes of NO_2 appeared. Then three drops of phosphate-free H_2O_2 were added and heating was completed in five minutes. The sample was diluted and an analysis for inorganic phosphorus was carried out.

Labile phosphorus was determined by placing an aliquot of the sample in 1 N H_2SO_4 and heating the mixture in a boiling water bath for 10-12 minutes. Upon cooling, the sample was analyzed for inorganic phosphorus.

Determination of the Position of the Ribose-Phosphate Linkage

The copper binding test developed by Caputto, et al. (1950) was carried out as described by Park (1952b). Two μM of sample in 1.5 ml. water were incubated with 3 mg. of copper phosphate and 0.5 ml. of 20 % trisodium phosphate for 1.5 hours with occasional mixing. After

centrifugation, 1 ml. of supernatant solution was mixed with 1 ml. of 0.5 % sodium diethyl dithiocarbamate. The copper complex was extracted with 7 ml. amyl alcohol, and measured in a Klett-Summerson colorimeter using a 540 mμ filter. A water blank as well as known positive and negative samples were included in each experiment.

Snake venom contains a 5'-nucleotidase. The action of this enzyme on the unknown compound was studied in an attempt to ascertain the position of the ribose-phosphate linkage. The enzyme was prepared by dissolving 1 mg. of the venom from Crotalus adamanteus in 100 ml. water. This venom was obtained in crystalline form from Ross Allen's Reptile Institute, Silver Springs, Florida. Two methods were used. According to the method described by Park (1952b), 1 to 2 μM sample was incubated at 37° C. with 0.4 ml. enzyme in 1 ml. of solution containing 0.1 M glycine, pH 8.5, and 0.01 M $MgCl_2$. The reaction was stopped by the addition of 0.1 ml. 40 % TCA at the end of the experiment and the mixture was analyzed for hydrolysis products. The other method used involved the following reaction mixture: 1 ml. 0.2 M ammonium hydroxide-ammonium acetate buffer, pH 9.2, 0.2 ml. 0.3 M $MgCl_2$, 0.4 ml. substrate (1-2 μM), and 0.4 ml. enzyme. Known positive and negative samples, as well as blanks were included in each experiment. The mixtures were incubated for 3 hours at 37° C. At the end of the incubation period, the reactions were stopped by the addition of 1 ml.

of 2.5 % TCA. The solutions were then analyzed for hydrolytic products by paper chromatography and spectrophotometry.

EXPERIMENTAL RESULTS

Pyrimidine Biosynthesis in Aerobacter aerogenes

In order to obtain evidence concerning the pathway of pyrimidine biosynthesis in bacteria, the analysis of genetically blocked organisms seemed to constitute a useful approach.

Production of Mutants

Attempts to produce mutants by ultraviolet irradiation were made with several organisms, using the methods of Davis (1948) and Lederberg and Zinder (1948). These methods involved irradiating the cells with ultraviolet light for a period long enough to kill 99.9% of the cells. This very high killing effect is necessary in order to insure that a high percentage of the survivors will be mutants. After incubating the irradiated cells in "complete" medium overnight, the cells were inoculated into minimal medium to which penicillin had been added in a concentration high enough to kill any proliferating cells. This treatment serves to remove the unwanted nonmutant cells which are able to grow in this medium, while the resting mutant cells remain viable. Obviously, the success of this method depends on the use of a penicillin sensitive organism.

Cells of Aerobacter cloacae were irradiated for various lengths of time. It was found that irradiation for at least 20 seconds was necessary

in order to produce the desired killing effect. However, this organism showed no sensitivity to penicillin even at 2,000 units per ml. Streptomycin was tested as an alternative agent, in an attempt to reduce the numbers of nonmutants. A. cloacae showed sensitivity to 30 units of streptomycin per ml. However, the mutants which resulted from this treatment were very unstable and reverted after a short period of time.

When cells of Serratia marcescens were irradiated 20 seconds, the desired killing effect was obtained. This organism also proved to be resistant to penicillin, but was sensitive to streptomycin at a level of 5 units per ml. All of the mutants which resulted in this study were amino acid mutants, many of which responded to several amino acids.

Because of the instability of the mutants produced with A. cloacae, and the non-specificity of the majority of the S. marcescens mutants, further use of these organisms was abandoned.

Attempts to produce mutants in Aerobacter aerogenes were successful. Irradiation of the cells for 10 seconds produced the desired killing effect. These cells showed a sensitivity to penicillin at 300-400 units per ml. Upon plating out of the penicillin-treated organisms, it was found that in the more successful experiments, approximately 30% of the colonies were mutants. Most of these mutants required amino acids, while an occasional purine or pyrimidine mutant was found. Approximately 200 pyrimidine-requiring mutants were finally collected.

The mutants of A. aerogenes were relatively stable. The few mutants which did show frequent reversion were discarded. Only those mutants which failed to revert after 18-24 hours incubation were retained for this study. In many cases, reversion of the mutants used did not occur after 48 hours and even longer periods of incubation.

Recombination Studies

The demonstration of recombination between certain pairs of mutants was attempted, in an effort to ascertain the nature of the genetic blocks through an analysis of the resultant progeny. Various modifications of the method used by Tatum and Lederberg (1947) to demonstrate recombination in Escherichia coli K-12 were used.

The nine mutants used in this study were grown in pairs in all possible combinations. The mutants all possessed double growth requirements: 3A, histidine and guanine; 15A, leucine and guanine; 17A, guanine and threonine; 18A, guanine and histidine; 10, histidine and leucine; 25, uracil and histidine; 26, uracil and histidine; 12, histidine and proline; 15, histidine and tryptophan.

After growing the cells in complete medium for four hours at 30° C., saline suspensions of the mutants were prepared which contained 10^9 cells per ml. One-tenth ml. of the suspensions was incubated singly and in combination with and without shaking at temperatures of 20° C., 30° C.,

and 37° C. The incubation period was varied from 5-10 minutes to 2-3 hours. The cell suspensions were then added to solid or liquid minimal media.

All experiments failed to show any appearance of growth, even after incubation for 5 days. It was finally concluded that if recombination does occur in A. aerogenes, the methods used were in some unknown way inadequate to detect this process, or the strain used was infertile.

Description of Pyrimidine Mutant Types

Upon examination of the pyrimidineless mutants collected, it was found that they could be classified into four distinct groups. Of the 200 pyrimidine-requiring mutants produced, two were of the type designated as "D"; three were of type "C"; eight were of type "B", and the remainder were type "A" mutants. It is difficult to explain why the majority of the pyrimidine mutants were of this latter type.

Table 1 shows the typical responses of these types to various supplements. It can be seen that mutants of types A, B, C, and D all utilized uracil, cytosine or the corresponding ribonucleosides and ribonucleotides for growth. None of the mutants responded to thymine, thymidine, thymidylic acid or methyl cytosine. The mutants of types B, C, and D utilized orotic acid to replace partially the requirement for uracil. The response to orotic acid by these mutants was not as complete

Table 1

Comparison of growth responses of four types of pyrimidine mutants and wild type cells in minimal medium containing various supplements. Turbidity measured in a Klett-Summerson Colorimeter with a 660 mμ filter after incubation at 30° C. with constant aeration for 22 hours.

Supplement 1 micromole per ml.	A 9	B 2	C 5	D 3	Wild Type
None	2	3	2	1	122
Aspartate	2	2	5	45	123
Ureidosuccinate	2	4	22	77	121
Orotate	3	29	71	110	127
Uracil	125	128	124	126	124
Uridine	138	138	138	137	139
Uridylate	142	144	140	142	138
Cytosine	123	130	128	128	126
Cytidine	135	137	134	137	134
Cytidylate	140	145	139	146	141

as was the response to the bases cytosine or uracil, or the corresponding nucleosides or nucleotides.

The type C mutants utilized ureidosuccinic acid for growth, in addition to orotic acid and uracil. Mutants of type D differ from the type C mutants in that they also utilized aspartic acid, and were able to utilize ureidosuccinic acid more effectively than the type C mutants. None of the mutants responded to succinate.

Addition of the various supplements to the minimal medium did not increase growth of the wild type culture except in the cases of the nucleosides and nucleotides.

The four types of uracil mutants responded almost identically to uracil, cytosine and the corresponding nucleosides and nucleotides. Response to uracil or cytosine was about equal in all cases. However, growth was better in the presence of equimolar concentrations of the nucleosides or nucleotides than with the corresponding free bases. These results are in agreement with observations made with other organisms (Loring and Pierce, 1944; Hammersten, et al., 1949, 1950).

The results in Table 1 show that the mutants respond equally well to uracil or cytosine; cytidine or uridine, uridylic acid or cytidylic acid. The response to a given nucleotide is greater in almost every case than to the corresponding nucleoside. The ease with which cytosine or its derivatives could be utilized instead of uracil or its related compounds

could indicate that the organisms possess active cytosine and cytidine deaminases, an indication substantiated by results obtained with paper chromatography.

One of the type D mutants shows a slight response to oxalacetate. However, because of the rapid decomposition of the oxalacetate, quantitative growth studies were difficult. In an attempt to maintain a supply of oxalacetate in the medium, 1.5 micromoles oxalacetate per ml. were added every two hours over a 24 hour growth cycle. An increased response has been observed when the oxalacetate is supplemented with glutamate, presumably due to the formation of aspartate. There is no response to glutamate alone, nor to pyruvate alone. Ureidoglutarate likewise produces no response when used alone, but increases of response have been noted when this compound is added either to oxalacetate or to aspartate. It has been suggested that ureidoglutarate may serve as a donor of the carbamyl group whereby aspartate is converted to ureidosuccinate in a transcarbamylation type of reaction (Wright, 1951).

All of the mutants responded similarly to uracil, cytosine, and the corresponding nucleosides and nucleotides, i.e., there were no individual variations in the response pattern.

The mutants were able to grow in media containing uracil levels as low as 0.1 to 0.5 micrograms per ml. (Table 2). These turbidities were read in a Coleman Nephelometer. When turbidities were measured

in an Evelyn colorimeter, levels of at least 1 microgram uracil per ml. were necessary in order to obtain appreciable readings. There was no appreciable difference in growth response within the range of 1-10 micrograms uracil per ml. (Table 3). However, beyond the level of 10 micrograms uracil per ml., the growth response increased steadily with increase in concentration until a level of about 90 micrograms per ml. was attained. Figure 1 shows a typical mutant response to increasing concentrations of uracil, using mutant A6. It can be seen that there was a steady increase in growth from 0.1 micromole to 0.82 micromoles (92 micrograms) per ml. There was a leveling off of growth response at this point. The addition of asparagine or amino acids to the medium did not increase the response to uracil. Aeration increased the growth response to uracil (and in all the media in general) to a marked degree. The growth response to uracil reached a maximum at about 18 hours.

There were considerable variations both within and among mutant types in the response to the postulated uracil precursors. Table 4 shows the variations of response of representative mutants to different concentrations of orotic acid. It can bee seen that mutant D3 utilized orotic acid almost as well as uracil. The other mutants showed a progressively poorer response. Mutants of type C showed less response than the type D mutants, while mutants of type B were able to utilize orotic acid only slightly. The type A mutants could not utilize orotic acid at all. The

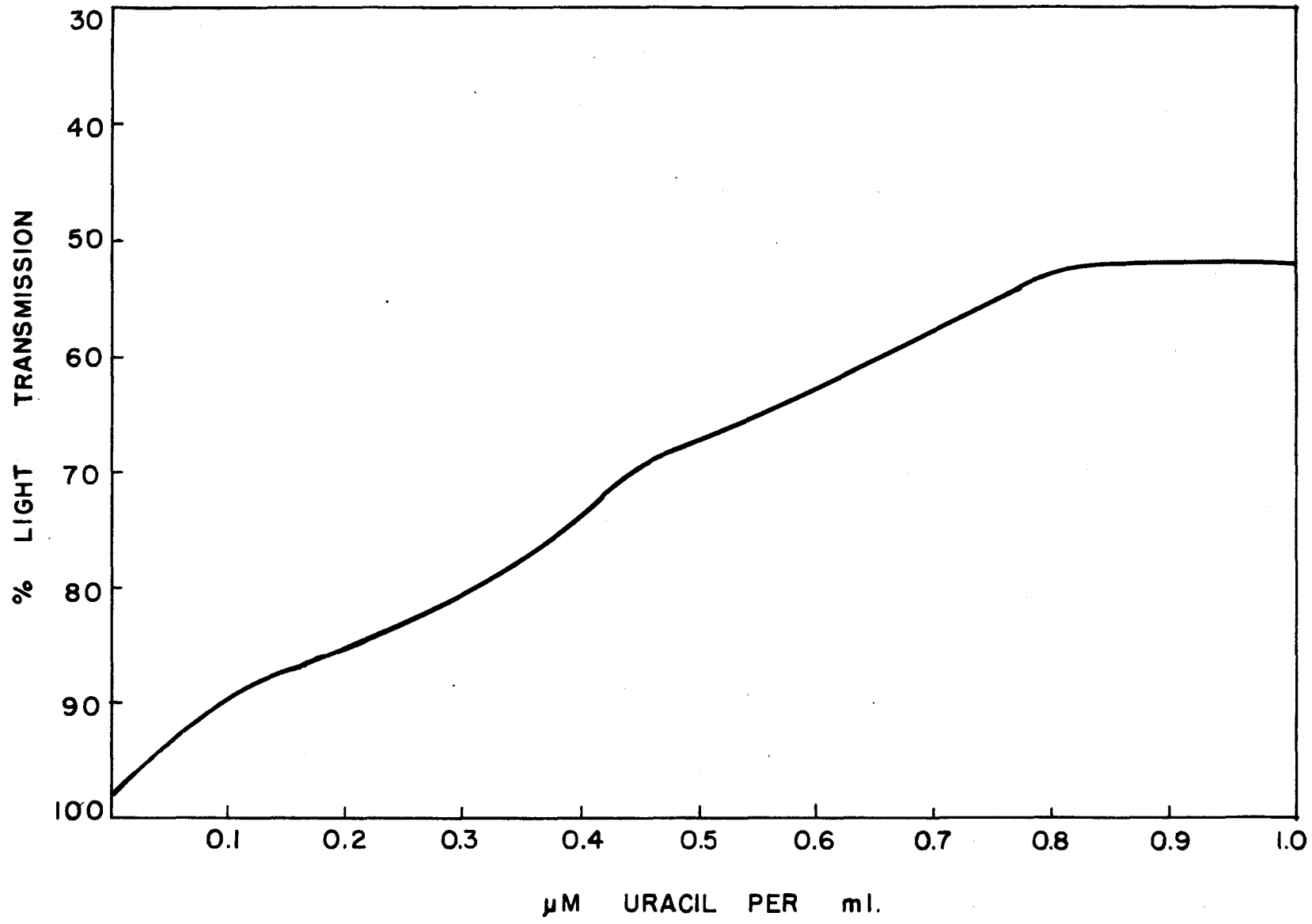


Fig. 1. Response of mutant A6 to increasing concentrations of uracil. Turbidities measured in an Evelyn colorimeter with a 620 mμ filter.

Table 2

Response of mutant A6 to low levels of uracil. Turbidity measured in Coleman Nephelometer with 660 mμ filter after incubation at 30° for 24 hours.

Micrograms uracil per ml.	Nephelometer reading
0.0	30
0.1	37
0.2	38
0.3	38
0.4	40
0.5	48
0.6	46
0.7	50
0.8	53
0.9	70
1.0	56
1.1	75
1.2	64
1.3	59
1.4	78
1.5	77

Table 3

Response of mutant A6 to low levels of uracil. Turbidities measured in Evelyn colorimeter with 620 mμ filter after incubation at 30° C. for 24 hours.

Micrograms uracil per ml.	Reading
0	100
0.5	95
1.0	91.5
1.5	87.0
2.0	86.0
2.5	85.0
3.0	83.5
3.5	86.5
4.0	80.0
4.5	85.0
5.0	83.0
5.5	85.0
6.0	87.5
6.5	88.0
7.0	88.0
7.5	88.0
8.0	87.0
8.5	89.0
9.0	81.0
9.5	86.0
10.0	85.0

Table 4

Growth responses of different mutants to various concentrations of orotic acid. Growth measured in Klett-Summerson colorimeter with 660 mμ filter after incubation for 24 hours with constant aerations at 30° C.

Mutant	(Micrograms per ml.) Orotic Acid Concentration 0	50	100	200
A-10	0	3	2	8
A-16	1	1	1	8
B-2	3	6	20	38
C-5	4	10	63	86
D-1	3	25	60	102
D-3	2	65	110	117

levels of orotic acid required to elicit a given degree of growth response are always higher than the concentrations of uracil needed to effect a comparable amount of growth. The maximum growth response, even of the best-responding mutant, D3, was less than the maximum growth response to uracil.

These variations in response to a given postulated precursor can also be observed with ureidosuccinic acid. Table 5 shows typical responses of different mutants to varying levels of ureidosuccinate. Mutant D3 again showed more response than any of the other mutants, although this response is lower than that shown by this organism in orotic acid and particularly uracil. Mutant D1 utilized all the postulated precursors, including ureidosuccinic acid, to a lesser extent than mutant D3. The type C mutants showed lower growth responses in the presence of ureidosuccinic acid than the type D mutants. Mutants of types B and A could not utilize ureidosuccinic acid for growth.

The responses of different organisms to increasing levels of aspartic acid are shown in Table 6. Only the type D mutants responded to this postulated precursor. Mutant D3 responded better than D1. However, the maximum response to aspartic acid was considerably below the response to ureidosuccinic acid, and particularly orotic acid and uracil. Mutant D3 also responded slightly to oxalacetic acid (Table 7).

There was a very short lag period in the growth response of the

Table 5

Growth responses of various mutants to different concentrations of ureidosuccinic acid. Turbidities measured in a Klett-Summerson colorimeter with a 660 mμ filter, after incubation for 24 hours at 30° C. with constant aeration.

Mutant	Concentration Ureidosuccinic Acid (Micrograms per ml.)						
	0	10	25	50	75	100	200
A-16	5	6	7	6	5	7	7
B-5	1	1	8	5	12	11	10
B-6	0	2	3	4	5	6	5
C-5	0	0	0	4	12	18	22
C-13	3	8	16	15	18	26	23
C-20	0	6	14	12	20	25	22
D-1	0	10	11	11	24	23	28
D-3	3	11	19	25	60	70	87

Table 6

Growth responses of different mutants to various concentrations of aspartic acid. Turbidities measured in a Klett-Summerson colorimeter with a 660 mμ filter after incubation at 30° C. with constant aeration for 24 hours.

Mutant	Concentrations of Aspartic Acid (Micrograms per ml.)			
	0	50	100	200
A-10	0	1	1	2
A-16	1	1	1	1
B-2	3	7	3	3
C-5	4	6	8	10
D-1	3	5	10	15
D-3	2	35	43	50

Table 7

Growth response of mutant D3 in minimal medium supplemented with L-aspartate and oxalacetate. Turbidity measured in a Klett-Summerson colorimeter with a 660 mμ filter after incubation at 30° C. with constant aeration for 24 hours.

Supplement 1.5 micromoles per ml.	Replicate Samples					Average
None	16	23	20	16	17	18
Oxalacetate[1]	25	41	33	35	37	34
L-aspartate	88	60	75	71	83	75

[1] Initial concentration, 1.5 micromoles per ml. The addition of 1.5 micromoles per ml. was made every two hours thereafter during the entire incubation period.

mutants to uracil, and in the growth of the parent culture in minimal medium (Table 8). However, the responses of the mutants to orotic acid, ureidosuccinic acid, and aspartic acid are consistently delayed responses, which occur after a lag of 14-16 hours and reach a maximum at about 24 hours. Table 8 illustrates these differences in response with data from a typical experiment in which Mutant D3 and the wild type organism were used.

Effect of Temperature on Pyrimidine Requirement

The effect of temperature on the pyrimidine requirement of different mutants was tested. Representative mutants were inoculated into minimal medium and minimal medium which contained a sub-optimal concentration of orotic acid (20 micrograms per ml.). The cultures were incubated for 24 hours at four different temperatures: 20° C., 30° C., 25° C., and 37° C. The results are summarized in Table 9. There was no growth in the minimal medium at any of the temperatures used. The cultures grown at 20° C. showed negligible growth in the tubes which contained orotic acid; growth at 25° C. was very slight. There was somewhat more growth at 30° C. and at 37° C. Growth at these temperatures was about equal. These results indicated that the pyrimidine requirement had not disappeared when the organisms were incubated at temperatures higher or lower than the 30° C. temperature

Table 8

Delayed responses of mutant D3 in minimal medium containing various supplements, as compared with response of parent organism in minimal medium. Turbidities measured in an Evelyn colorimeter with a 620 mμ filter. Cultures incubated at 30° C. with constant aeration.

	Mutant D3					Parent Organism
	Supplements (1.5 μM per ml.)					
Hours of Incubation	None	L-Aspartate	Ureido-succinic Acid	Orotic Acid	Uracil	None
0	98	90	91	99	100	100
6	98	90	91	98	95	90
8	98	90	91	98	88	81
10	97	90	91	97	86	81
12	97	90	90	90	79	74
14	97	90	89	88	68	67
16	97	88	88	81	61	62
18	98	88	86	76	56	58
20	97	87	83	71	54	54
24	96	84	77	67	44	47

Table 9

Relative growth responses of different mutants in minimal medium and in minimal medium containing 20 micrograms orotic acid per ml. at different temperatures. Cultures were incubated 24 hours.

	B 2		B 4		B 6		C 5		D 1	
					Supplements					
		Orotic		Orotic		Orotic		Orotic		Orotic
Temperature	None	Acid	None	Acid	None	Acid	None	Acid	None	Acid
20° C.	-	-	-	-	-	-	-	-	-	-
25° C.	-	(+)	-	(+)	-	(+)	-	(+)	-	(+)
30° C.	-	(+)	-	++	-	+	-	(+)	-	+
37° C.	-	(+)	-	++	-	+	-	(+)	-	+

(+) = Very Faint Turbidity
\+ = Faint Turbidity
++ = Fair Turbidity

usually employed. Houlahan and Mitchell (1947) found that two pyrimidine-requiring mutants of Neurospora almost lost the pyrimidine requirement exhibited at 35° C., when the cultures were incubated at 25° C.

Pyrimidine Utilization Studies

The ability of uracil to serve as a sole source of a) carbon, b) nitrogen or c) carbon and nitrogen was studied. Modifications of the minimal medium were prepared, in which 0.1 % uracil replaced the original a) glucose and sodium citrate content b) ammonium sulfate content and c) glucose, sodium citrate, and ammonium sulfate content. The parent culture and a representative mutant were inoculated into tubes containing these different media. After incubation for 18 hours, there was no growth response in any of the tubes. Table 10 shows the growth responses of these organisms in these media after incubation for 42 hours. These data show that the organisms were unable to utilize uracil as a sole source of carbon, or as a sole source of carbon and nitrogen. The growth responses of the parent and mutant organisms were almost identical in the medium containing uracil as the only nitrogen source. Apparently, uracil was utilized to some extent as a nitrogen source. The delay in the growth response when uracil served as the only source of nitrogen would indicate that the breakdown of uracil is catalyzed by adaptive enzymes. Lara (1952b) observed a similar response in Corynebacteria and Nocardia

Table 10

Growth responses of parent and mutant organism in minimal medium in which 0.1 per cent uracil serves as sole source of carbon, nitrogen, or carbon and nitrogen. Turbidity measured in Klett-Summerson colorimeter with 660 mμ filter after incubation for 42 hours, at 30° C.

	0.1 per cent Uracil Sole Source of		
Organism	Carbon	Nitrogen	Carbon and Nitrogen
Parent Culture	0, 1	15, 15	0, 0
A9	1, 1	16, 14	1, 1

species, when uracil or thymine served as the sole source of nitrogen, carbon and energy.

Syntrophy Studies

If mutants are blocked at different points, it would be expected that one mutant should accumulate a metabolic intermediate which could be utilized by another mutant blocked earlier in the reaction sequence. The utilization of this intermediate would thus enable the second mutant to grow.

Mutants of type A were studied in an attempt to ascertain whether there were any differences in the position of the enzymatic block possessed by members of this group. None of these organisms responded to orotic acid, while all responded to uracil, to an almost identical extent. It seemed probable that these organisms would either accumulate orotic acid or some unknown intermediate lying between orotic acid and uracil, depending on the position of the block. Six type A mutants, A6, A9, A27, A22, A32, and A55 were studied. Attempts were made to demonstrate syntrophy by inoculating these organisms in pairs and trios into tubes of minimal medium. The mutants were also inoculated in adjacent streaks on solid minimal medium. No growth could be noted. In order to insure a small amount of growth, with subsequent production of intermediate(s), the experiments were repeated using liquid and solid minimal media

supplemented with 0.01 % casein hydrolyzate. Again, syntrophy was not observed inasmuch as no increase in growth above controls could be noted.

Similar tests for syntrophy were made using combinations of type A mutants with the orotic acid requiring mutants, D 1, B 2, D 3, B 4, C 5, and B 6. The orotic acid requiring mutants listed were also tested in various combinations with one another. All the experiments gave inconclusive results.

Failure to observe syntrophy between or among types could have been due to a lack of permeability of the intermediate(s) formed. Lack of syntrophy among members of a general type could indicate that the organisms were all blocked at the same point.

Studies of Metabolic Conversion Products

Qualitative and quantitative estimations of pyrimidine compounds produced by the organisms in the presence of different supplements were made through paper chromatography and spectrophotometry.

Various solvents were tested for their ability to separate the various pyrimidine compounds. N-butanol, n-propanol, ethanol, n-propanol-H_2O (3:1), ethanol-water (4:1), butanol-water (3:1), n-butanol-ethanol-H_2O (4:1:1) and n-butanol-glacial acetic acid-H_2O (4:1:1) were studied. Of these, n-butanol-glacial acetic acid-H_2O proved to be the

superior solvent for separation of the compounds under study. In some cases, ethanol-water (4:1) was also used. Table 11 shows average R_f values of different pyrimidine compounds in butanol-acetic acid-H_2O solvent. Absorption maxima are also shown. Typical migrations of the compounds in butanol-acetic acid-H_2O solvent are shown in Figure 2.

Products Accumulated in the Presence of Pyrimidine Compounds

When mutants A6 and B11 were grown on minimal medium containing 100 micrograms uracil per ml., no uridine, uridylic acid or other pyrimidine compounds could be detected in the supernatant. The small amount of uracil which disappeared was apparently converted to higher intermediates in minute quantities which were probably almost immediately utilized for cell nucleoprotein formation. Some of the uracil could have been catabolized, since this organism is able to utilize uracil to a limited extent as a nitrogen source. However, it is doubtful whether uracil is actually used by this organism as a nitrogen source except when there are no other nitrogen sources present in the medium.

The wild type organism when grown on minimal medium containing 100 micrograms cytosine per ml. accumulated 70-80 micrograms uracil per ml. in the supernatant. The mutant organisms (A6 and B11) accumulated similar amounts of uracil when grown in minimal medium containing 100 micrograms cytosine per ml. No cytosine remained.

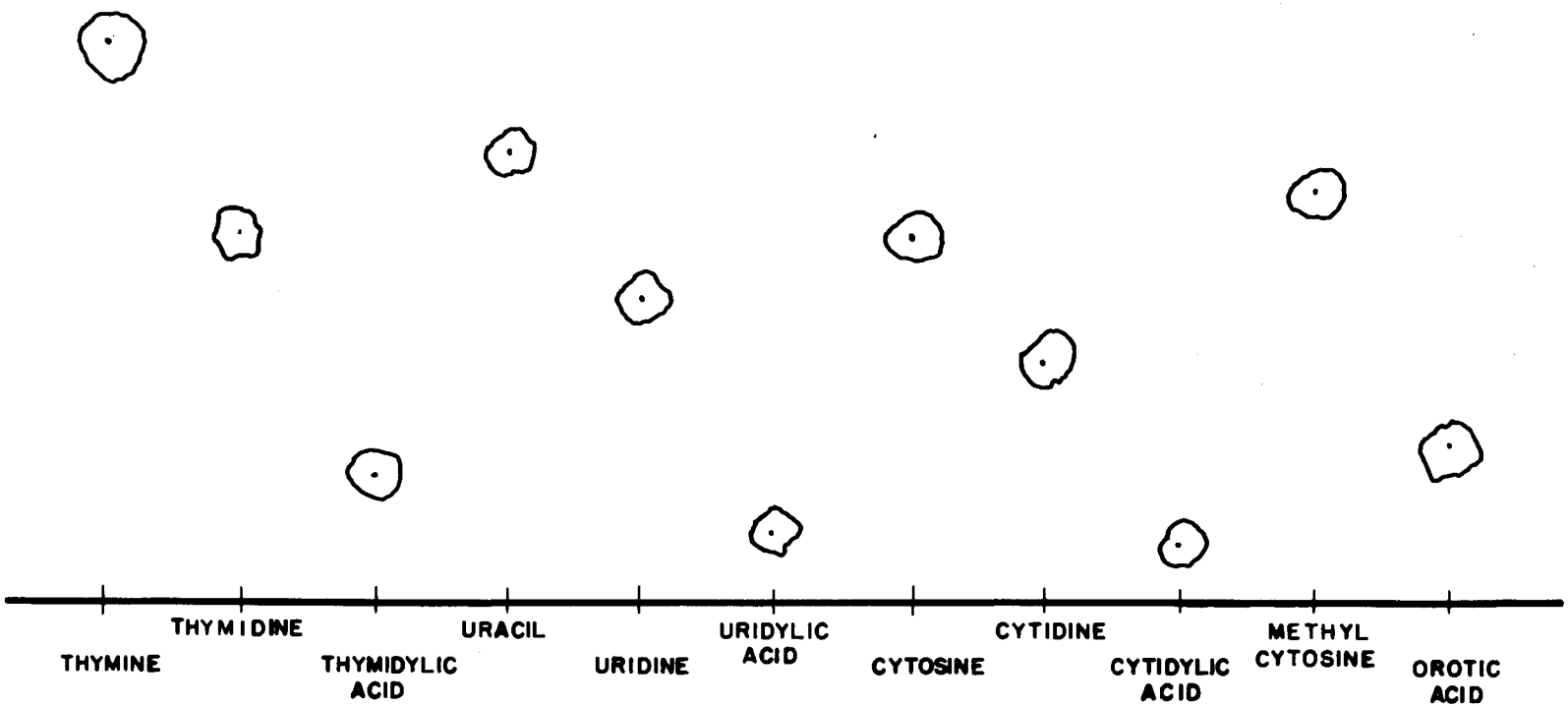
Table 11

R_f values and absorption maxima of various pyrimidine compounds.

Compound	Average R_f values[1]	Absorption maximum
Uracil	0.49	260
Uridine	0.33	262[2]
Uridylic Acid	0.07	260
Cytosine	0.40	275
Cytidine	0.26	280
Cytidylic Acid	0.06	275-280
Thymine	0.61	263
Thymidine	——	265
Thymidylic Acid	0.13	270
Methyl Cytosine	0.45	——
Orotic Acid	0.17	280-285
Unknown Compound	0.07	260-265[2]

[1] In butanol-acetic acid-H_2O solvent (4 : 1 : 1 by volume)
[2] In 0.1 N HCl

Fig. 2. Relative migration of different pyrimidine compounds in n-butanol-acetic-acid-H_2O solvent.


THYMINE
THYMIDINE
THYMIDYLIC ACID
URACIL
URIDINE
URIDYLIC ACID
CYTOSINE
CYTIDINE
CYTIDYLIC ACID
METHYL CYTOSINE
OROTIC ACID

Studies of the wild type organism (Shapiro, et al., 1953) showed that no conversion products could be detected when the parent organism was grown in the presence of thymine. None of the mutant types utilized thymine, thymidine, or thymidylate for growth.

Methyl cytosine could not replace the mutant requirement for cytosine or uracil. Nineteen representative mutants of all four types were inoculated into minimal medium containing 40 micrograms methyl cytosine per ml. There was no response after 46 hours incubation. This compound could not be utilized by the mutants; neither did it inhibit the growth of the nineteen representative mutants when present in the medium along with uracil or cytosine. The wild type organism when grown on minimal medium supplemented with 100 micrograms methyl cytosine per ml. accumulated 60-70 micrograms thymine per ml. of supernatant.

When grown in the presence of uridine or uridylic acid in concentrations of 100 micrograms per ml., respectively, the wild type organism and mutant A9 produced uracil. The original uridine and uridylic acid appeared to be completely metabolized.

Mutant A6 and the wild type organism accumulated uracil, uridine, and uridylic acid when grown in a minimal medium supplemented with cytidine. When grown on minimal medium containing 100 micrograms cytidylic acid per ml., the wild type organism and mutant D3 accumulated

uracil only in the medium.

Products Accumulated in the Presence of Postulated Precursors

Orotic acid was converted to uracil by the wild type organism (Shapiro, et al., 1953) and by mutants of types B, C and D. The type A mutants when streaked adjacent to mutants of types B, C, or D on a minimal agar medium supplemented with 100 micrograms orotic acid per ml. showed a definite growth response on the side of the streak near the mutant which utilized orotic acid. Similar effects have been noted when the mutants were streaked adjacent to the wild type organism, on minimal medium, or when mutants of types A or B were streaked adjacent to mutants of types C or D on minimal medium supplemented with ureidosuccinic acid. This effect has also been observed when mutants of types A, B, or C are streaked adjacent to a type D mutant on a minimal medium supplemented with aspartic acid. These effects are presumably due to excreted uracil.

Growing cultures of the wild type as well as the mutant organisms of types C and D converted ureidosuccinic acid to orotic acid and uracil. When grown in minimal medium containing 100 micrograms ureidosuccinic acid per ml. the wild type organism accumulated at least 2.5 micrograms orotic acid per ml. and 3.5 micrograms uracil per ml. of supernatant.

When grown in minimal medium supplemented with 100 micrograms aspartic acid per ml., both the wild type organism and the type D cells produced traces of cytosine and uracil. No orotic acid was detected under these conditions.

Because of the low growth response to oxalacetate, the demonstration of the conversion of this compound to uracil was not attempted.

Accumulation of Uridylic Acid

It was early observed that an unknown pyrimidine compound accumulated in the cells as well as in the medium in the presence of cytidine or orotic acid. It did not accumulate when the cells were grown in a peptonized milk medium which contained no pyrimidine supplements. Spectrophotometric studies showed that it had an absorption maximum of 260-265 mμ in 0.1 N HCl. The absorption spectrum was very similar to uracil. Chromatographic studies showed that the compound had an R_f value in butanol-acetic acid-H_2O similar to uridylic acid. However, it was not certain whether the substance was uridine-3'-phosphate or uridine-5'-phosphate. It was also not certain whether the substance was a mono-, di-, or triphosphate.

Table 12 shows the amounts of uridylate accumulated by the wild type organism and different mutants in various media. The amounts of uridylate accumulated in the presence of cytidine varied from 0.076 mg.

Table 12

Amounts of uridylate accumulated in the cells when different organisms were grown in different media. Cultures were incubated at 30° C. with constant aeration for 20-24 hours.

Organism	Supplement[1]	Cell Yield[2]	μM Uridylate	Mg. Uridylate	Mg. per Gram Cells[2]
A6	15μg cytidine per ml.	10 grams	3.3	1.07	0.107
A6	15μg cytidine per ml.	14 grams	13.4	4.34	0.310
A9	25μg cytidine per ml.	38 grams	8.9	2.89	0.076
D3	25μg cytidine per ml.	48 grams	8.5	2.75	0.057
D3	25μg cytidine per ml.	44 grams	120	38.9	0.884
Wild Type	25μg cytidine per ml.	46 grams	68.8	22.3	0.484
Wild Type	Peptonized Milk Medium	81 grams	—	—	—

1 Minimal medium was used, containing various amounts of cytidine, with the exception of the last experiment.

2 Calculations based on cell wet weight.

to 0.884 mg. per gram (wet weight) of cells.

Hydrolysis of the compound was accomplished only after autoclaving for 2 hours in 8 N HCl at 15 pounds pressure. Uracil was detected in the reaction mixture along with traces of the unhydrolyzed original compound following this treatment. The Mejbaum (1939) ribose test (using varying amounts of uridine-3'-phosphate as standards) showed that approximately one mole of ribose was present per mole of unknown compound. Calculations were made using the E_m value for uracil at 260 mμ, 1×10^4.

Phosphate determinations made according to the Fiske and SubbaRow (1925) method showed approximately one mole of phosphorus per mole of unknown compound. The compound was thus established as a uridylic acid, but the position of the ribose-phosphate linkage was not as yet established.

The copper binding test was first used in an attempt to determine the position of the linkage. Compounds which contain two hydroxyl groups in the cis-position are able to form a copper complex in the presence of cupric ions; uridine-5'-phosphate contains such a glycol grouping, while uridine-3'-phosphate does not. This test, however, gave inconclusive results since the solutions of the compound obtained by elution of paper chromatograms as well as filter paper control solutions gave very high unreliable Klett readings. These readings, in a typical

experiment, were 670 for the unknown solution (2 micromoles), and 610 for the filter paper control. Two micromoles of pure adenosine-5'-phosphate gave a reading of only 65 in this experiment. These high readings were apparently due to impurities from the filter paper which were able to bind copper.

The supernatant solution resulting from the lead sulfide precipitation in the procedure for isolation of the uridylic acid was then studied, without subjecting it to chromatographic purification. This was done in order to eliminate the interference by the filter paper. The results obtained with these solutions indicated that the unknown compound was able to bind an amount of copper similar to that bound by equivalent concentrations of adenosine-5'-phosphate. Two micromoles of the unknown compound and adenosine-5'-phosphate gave readings of 30 and 32, respectively, with a control reading of 0. However, the procedure employed for the isolation of the pyrimidine fractions from the cells also served to isolate certain other classes of compounds which could contain cis-hydroxyl groups, e.g., sugars.

An alternative method was used in an attempt to obtain more conclusive results. A 5'-nucleotidase found in snake venom was tested for its ability to hydrolyze the unknown compound. Two methods were employed. In both methods, solutions of the unknown compound, uridine-5'-triphosphate, uridine-3'-phosphate and adenosine-5'-phosphate

(containing one micromole respectively) were each incubated at 37° C. with 0.4 ml. of the enzyme solution. Chromatographic study of the solutions at the end of the incubation period showed that no hydrolysis of the unknown compound occurred using either method. However, uridine and adenosine were produced from the uridine-5'-triphosphate and adenosine-5'-phosphate, respectively. These results indicated that the compound under study was not uridine-5'-phosphate, but probably uridine-3'-phosphate (uridylic acid b). A solution of the compound gave an absorption curve almost identical to that of a standard uridine-3'-phosphate solution (Figure 3).

Attempts to isolate the compound as the free uridylate or as the dibrucine derivative were unsuccessful.

Resting Cell Experiments

The conversion of aspartic, ureidosuccinic, and orotic acids to uracil, as well as the conversion of ureidosuccinic acid to orotic acid was demonstrated with growing cells. The demonstration of these reactions with resting cells was also attempted. Various amounts of inocula were added to a reaction mixture containing 0.07 M phosphate buffer, M/36 glucose, and 1 micromole of substrate in a total volume of 25 ml. The substrates tested were orotic acid, ureidosuccinic acid, and aspartic acid. After incubation on a shaker for 20-24 hours, the cells

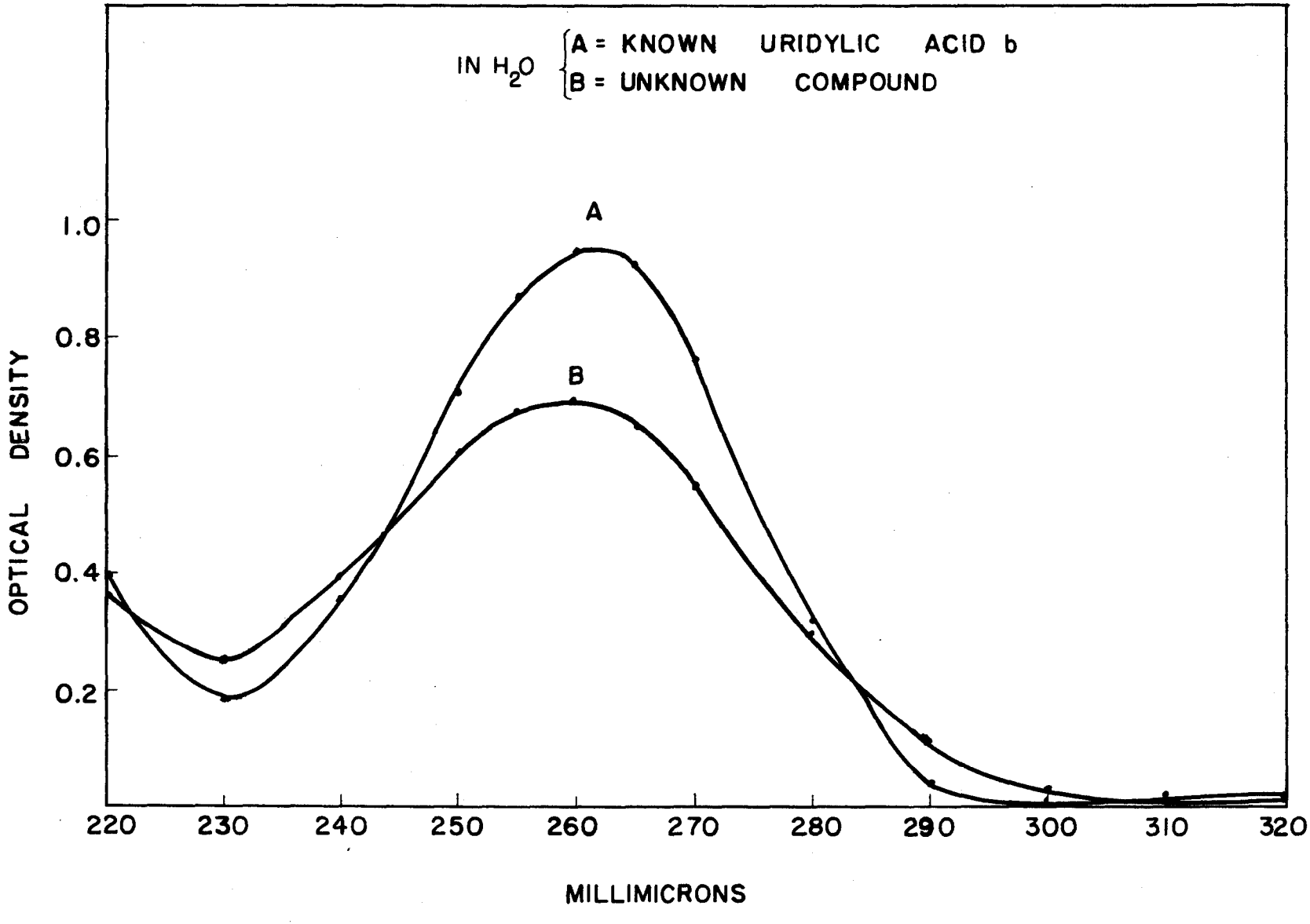


Fig. 3. Ultraviolet absorption spectra of solutions of uridylic acid b and the unknown compound.

were removed by centrifugation and the resultant supernatants were studied chromatographically. No uracil or orotic acid was produced from any of the substrates under the conditions employed.

Cell-free Extract Experiments

The demonstration of these reactions was also attempted with cell-free extracts of the wild type organism. Both the Dubnoff metabolic shaker and the Warburg respirometer were employed in these studies.

Dubnoff metabolic shaker studies

Experiments were carried out with a cell-free extract prepared from cells grown on minimal medium supplemented with 100 micrograms orotic acid and 100 micrograms ureidosuccinic acid per ml. One ml. of the extract was added to each flask containing 8 μM substrate, 0.1 % glucose, and 10 μM ATP in 2 ml. of 0.130 M phosphate buffer. The substrates were neutralized to pH 7 before use. The substrates tested were oxalacetate, aspartate, aspartate + ureidoglutarate, ureidosuccinate, and orotate.

The flasks were incubated in the Dubnoff metabolic shaker at 30° C., and samples taken at intervals were studied chromatographically. The chromatograms showed diffuse purple streaks which extended up from the base line. These streaks could have been due to ATP or its

degradation products, since ATP had been added to every flask mixture, and all adenine compounds absorb in the ultraviolet region.

In order to determine whether this streaking was due to the ATP added, the extract was incubated with substrate both in the presence and in the absence of 10μM ATP. Five-tenths ml. of the extract was added to each flask containing 10 micromoles substrate; the reaction mixture was made up to 2 ml. with 0.130 M phosphate buffer. The substrates studied were orotate and ureidosuccinate. Aliquots of the mixtures taken at intervals during the incubation period were studied chromatographically. The chromatograms showed generalized streaks as before. Since even the chromatograms of the mixtures without ATP showed streaking, it was thought that at least some of these diffuse spots must be due to cell protein.

The mixtures which did not contain ATP were thereupon treated with one volume of 2.5 % TCA and heated slightly to facilitate precipitation of the protein. The supernatant solutions were studied chromatographically. The solutions which had contained orotate as substrate showed uracil spots, while the solutions which had contained ureidosuccinate as substrate showed orotic acid and uracil spots.

In subsequent studies, 0.2 ml. of the extract was added to each flask rather than 0.5 ml. or more, in an effort to reduce the amount of protein and possible cell autolysis. No ATP was added. The aliquots of

the solutions were deproteinized before studying them chromatographically. The control solution, the solution containing 40 M ureidosuccinate, and the solution containing 40 M orotate all showed uracil spots, both the control and the ureidosuccinate solutions showed orotic acid spots. The spots were cut out, eluted, and studied spectrophotometrically. The amounts of orotic acid and uracil produced were calculated from optical density measurements. The results are tabulated in Table 13. There was a 2.10 % conversion of ureidosuccinate to uracil, and 0.18 % conversion of orotate to uracil, on a molar basis. There was a 0.43 % conversion of ureidosuccinate to orotate.

Warburg respirometer studies

Warburg respirometer studies were made to determine whether any CO_2 evolution due to decarboxylation of orotic acid to uracil occurred. Experiments were carried out aerobically and anaerobically. Cell-free extracts prepared from cells grown in minimal medium supplemented with 100 micrograms orotic acid per ml., and from cells grown on the peptonized milk medium were used. Both extracts showed very small amounts of CO_2 evolution in the presence of 4 micromoles orotate or 40 micromoles ureidosuccinate. These small amounts of CO_2 evolution were noted in both aerobic and anaerobic experiments. The flask contents were deproteinized at the end of the Warburg experiments, and analyzed chromatograph-

Table 13

Orotic acid and uracil produced by a cell-free extract of wild type organism when incubated at 30° C. in Dubnoff Metabolic Shaker in presence of ureidosuccinate and orotate (40 μM per flask), pH 7.0

Substrate	Micrograms Orotic Acid Produced	Per Cent Conversion	Micrograms Uracil Produced	Per Cent Conversion
None	2	——	166	——
Ureido-succinate	29	0.43	259	2.10
Orotate	—	——	174	0.18

ically for the production of uracil and orotic acid. The results showed that uracil was produced in amounts so small that it could account for only a part of the CO_2 evolution. The remaining CO_2 must have arisen through catabolism of the substrate, or through non-specific effects. The CO_2 evolution ranged from 0-60 microliters above endogenous values.

Aerobic experiments. Table 14 shows the results of a typical aerobic Warburg experiment. No ATP was added. The substrates employed were 4 micromoles orotate, and 40 micromoles ureidosuccinate. The earlier use of 40 micromoles orotate was abandoned, because much of this substrate remained undissolved at this concentration. It was found that the limit of solubility of orotate in 2.3 ml. solution was approximately 4 micromoles. In this experiment, no orotic acid could be detected when aliquots of the ureidosuccinate mixture were studied chromatographically. However, uracil and a compound identified as uridine appeared on chromatograms made from the orotate, ureidosuccinate, and control mixtures (Table 14). The uracil formed in these respective mixtures would account for 11, 13, and 9 microliters of CO_2, if it all arose through the decarboxylation of orotate, during the experiment. The actual CO_2 evolution in these respective flasks was 0, 61.1, and 18.5 microliters. The CO_2 which is unaccounted for could have been produced through catabolism of the substrate, or through various non-specific effects. The zero CO_2 evolution in the orotate flask is difficult to explain, since the uracil

Table 14

Aerobic production of uracil and uridine by a cell-free extract of wild type organism in presence of orotate (4μM) and ureidosuccinate (40μM). Incubated at 30.4° C. in Warburg Respirometer. pH 7. Total volume 2.3 ml.

Substrate	Microliters CO_2 Evolved	Micrograms Uracil Produced	Per Cent[1] Conversion	Micrograms Uridine Produced	Per Cent[1] Conversion
None	18.5	46	——	80	——
Ureido-succinate	61.1	63	0.38	136	0.58
Orotate	0	55	2.00	89	0.93

[1] Molar basis

formation in that flask should have produced 11 microliters CO_2. There is of course the possibility that the uracil in the flask was produced as a result of degradation of the protein in the cell-free extract. The higher amounts of uracil and uridine formed in the ureidosuccinate mixtures as compared with the orotate mixtures could be due to the higher concentrations of ureidosuccinate used. The conversion of ureidosuccinate to uracil in this experiment was 0.38 %, while the conversion of orotate to uracil was 2 % (on a molar basis). Ureidosuccinate was converted to uridine in the amount of 0.58 %, while about 0.93 % of the orotate appeared to be converted to uridine.

Anaerobic experiments. Table 15 shows the results of a Warburg experiment carried out in an atmosphere of nitrogen. Ten micromoles ATP were added to each flask. There was no CO_2 evolution in the control flask, while 20 microliters CO_2 were evolved from 40 micromoles ureidosuccinate, and 44 microliters CO_2 were evolved from 4 micromoles orotate. The uracil formed in this experiment in the control, ureidosuccinate, and orotate mixtures could account for 23, 27, and 24 microliters CO_2 if the uracil had all been formed by decarboxylation of orotate during the experiment. There was a 0.43 % conversion of ureidosuccinate to uracil, and a 0.68 % conversion of orotate to uracil. The chromatograms showed bands in the usual orotate region. These bands were cut out, eluted, and the solutions were studied spectrophotometrically.

Table 15

Anaerobic production of uracil and guanine by a cell-free extract of wild type organism in presence of orotate (4 μM) and ureidosuccinate (40 μM), pH 7.0. Incubated at 30.4° C. in Warburg Respirometer. Total volume 2.3 ml.

Substrate	Microliters CO_2 Evolved	Micrograms Uracil Produced	Per Cent Conversion	Micrograms Guanine Produced[1]
None	0	117	——	584
Ureido-succinate	20	136	0.43	636
Orotate	44	120	0.68	427

[1] 10 micromoles adenosine triphosphate were present in each flask (including control).

The solutions gave an absorption maximum at 250 mμ. Since orotic acid gives an absorption maximum of 280 mμ, these solutions probably did not contain orotic acid. The only purine or pyrimidine which exhibits an absorption maximum at 250 mμ and which shows a migration in butanol-acetic acid-H_2O solvent similar to orotic acid seems to be guanine. The amounts of this compound which were formed are tabulated in Table 15. The source of the guanine could have been the ATP, present in 10 micromole amounts in each flask. It is possible that the enzyme preparation contained an enzyme which catalyzed the conversion of adenine to guanine.

Miscellaneous Mutant Types

Attempts to produce a mutant which would respond to cytosine but not to uracil were unsuccessful. Attempts to produce mutants which would respond only to pyrimidine nucleosides or nucleotides also failed. However, considerable numbers of purine and other interesting mutants were collected. These mutants were tested for their ability to utilize an enzymatic digest of calf thymus (desoxyribonucleic acid). None of the pyrimidine mutants were able to utilize this mixture for growth. While all of the purine mutants responded to this digest, some of the other mutants isolated responded to the digest only. They were unable to grow in minimal medium supplemented with the free purine or pyrimidine

bases, the corresponding ribonucleosides or ribonucleotides, or to any of the desoxyribonucleotides. Apparently, they required a dinucleotide or more complex compound present in the digest. Fractionation of this digest is now in progress, so that tests can be made to determine specifically which compound(s) in this partially hydrolyzed desoxyribonucleic acid mixture the "digest" mutants require.

DISCUSSION

Frequency of Occurrence of Different Mutant Types

It is difficult to explain why the majority of the mutants produced required amino acids. Apparently, enzymes involved in amino acid metabolism were affected by ultraviolet light more frequently than other enzymes. This could be due either to their presence in numbers higher than other enzymes or to their greater vulnerability to ultraviolet light. The possibility that amino acid enzymes are present in larger numbers than other types of enzymes seems probable in view of the fact that protein consists of more than twenty amino acids.

It is equally difficult to explain why the majority of the pyrimidine mutants collected were of type "A". It seems probable that the enzyme(s) involved in the reaction(s) between orotic acid and uracil are more easily affected by ultraviolet light than the enzymes involved in the preceding steps of the proposed reaction sequence.

Unfortunately, the mechanism of mutation resulting from ultraviolet irradiation is as yet unknown. Until this process is more clearly understood, any explanation regarding frequency of production of any particular mutant type is mere conjecture.

Postulated Precursors of Uracil

The observations made in this study are consistent with the

following postulated reaction sequence:

$$\text{Oxalacetate} \xrightarrow{D} \text{Aspartate} \xrightarrow{C} \text{Ureidosuccinate} \xrightarrow{B}$$

$$\text{Orotate} \xrightarrow{A} \text{Uracil.}$$

This does not imply necessarily that these reactions represent single enzymatic steps in the biosynthesis of uracil. An organism which responds to aspartate always shows response to those compounds which are beyond it in this proposed reaction sequence. Similarly, if an organism does not respond to orotate, neither will it respond to those substances which come before it.

Oxalacetate has been suggested as the most likely elementary precursor of the pyrimidines (Mitchell and Houlahan, 1947; Buchanan and Wilson, 1953). Aspartate has also been postulated as a probable elementary precursor (Mitchell and Houlahan, 1947; Lagerkvist, et al., 1951).

Wright, Valentik, et al. (1950) failed to obtain a response to oxalacetate or to aspartate by Lactobacillus bulgaricus 09. However, this lack of response could have been due to the low levels of oxalacetate and aspartate used. They reported that "at least 100 micrograms per tube" was used. The volume of liquid medium used was not given. In these experiments with A. aerogenes, at least 50 micrograms aspartate per ml. was necessary in order to elicit an appreciable growth response on the part of the type D mutants. This would be equal to 500 micrograms aspartate per tube. In order to obtain an appreciable growth response in the

presence of oxalacetate, a concentration of at least 200 micrograms per ml. was maintained. This would be equivalent to 2000 micrograms or 2 mg. oxalacetate per tube.

The relative ability of the type D mutants to utilize the various compounds decreases in this order: uracil, orotate, ureidosuccinate, aspartate, oxalacetate. The type C mutants respond better to uracil than to orotate which in turn supports better growth than ureidosuccinate. The type B mutants do not utilize orotate for growth as well as uracil.

It is interesting to note that the type D mutants utilize orotate almost as well as uracil. These organisms utilize orotate and ureidosuccinate better than the type C mutants. Similarly the type C mutants respond better to orotate than the type B mutants. Thus it appears than an organism blocked at any given point utilizes precursors beyond that block with an increasing facility.

There is some variation within mutant types as to the degree of response to the different postulated precursors. This would indicate that the mutants vary in the degree of enzymatic block as well as in type of enzymatic block.

Wright and Miller (1952) reported that uracil will replace the orotic acid requirement of Lactobacillus bulgaricus 09 only after prolonged incubation. This observation is difficult to reconcile with the results reported here with A. aerogenes, since uracil elicits a greater response

than orotate or any of the other postulated precursors. The response of the mutants to any precursor before uracil occurs after a lag of 14-16 hours, while growth on uracil is usually initiated between 6-8 hours (Table 8). These observations are similar to those of Back and Woods (1953) who observed an increased lag phase of 7 to 20 hours when a uracil requiring mutant of E. coli (B. coli) was grown in a medium containing ureidosuccinate instead of uracil.

Utilization of Pyrimidine Bases, Nucleosides and Nucleotides

The nucleosides, and particularly the nucleotides, elicited a greater growth response than the free pyrimidine bases. These results are analagous to observations made by Loring and Pierce (1944), and Hammersten, et al. (1949, 1950), working with other organisms. The greater response in the presence of nucleosides or nucleotides would indicate that these compounds are more directly available to the organism than the free pyrimidine bases. This more direct utilization could be due to greater solubility of these compounds as compared with the free bases. It could also be due to a greater permeability of the cell wall to nucleosides or nucleotides, than to the free bases. The greater response to nucleosides or nucleotides could also result if these compounds lie progressively further on in the reaction sequence, beyond the free bases. Their utilization would thus require fewer enzymatic steps than would be

involved in conversion of the free base into cell nucleic acid.

Attempts to Ascertain Nature of Enzymatic Blocks

Several approaches were used in an endeavor to ascertain the nature of the enzymatic blocks possessed by the four mutant types.

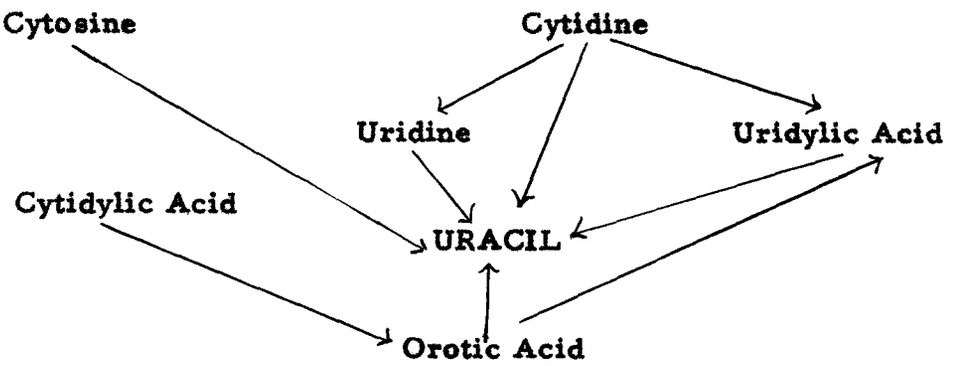
Houlahan and Mitchell (1947) found that the pyrimidine requirement exhibited by two mutants of Neurospora crassa when grown at 35° C. almost disappeared when the cultures were incubated at 25° C. Apparently, the original temperature optimum of an enzyme had been lowered in the mutation process, so that the mutant appeared to be phenotypically different from the parent organism when grown at 35° C. It was thought that perhaps the pyrimidine requirement of the Aerobacter aerogenes mutants might also vary depending on the temperature of incubation. However, contrary to the observations of Houlahan and Mitchell (1947) with N. crassa, the mutants of A. aerogenes showed no change in pyrimidine requirement at different temperatures. Growth at temperatures higher or lower than the 30° C. temperature usually employed did not result in a loss of the pyrimidine requirement.

Attempts were also made to demonstrate the accumulation of an intermediate previous to a given block. However, syntrophy did not occur either when mutants of different types or when mutants within a type were grown in minimal medium in pairs or trios. It would be expect-

ed that the type "A" mutants would accumulate orotic acid, since they can not utilize this compound, but respond only to the free bases or corresponding nucleosides or nucleotides. Nevertheless, chromatographic studies failed to show any accumulation of orotic acid when mutants of type A were grown in minimal medium in the presence of 100 micrograms uracil per ml. It is possible that the orotic acid formed was catabolized so rapidly that it escaped detection.

Metabolism of Pyrimidine Compounds

The following reactions appear to occur during growth in A. aerogenes:



It can be seen that uracil occupies a central position in pyrimidine metabolism in this organism.

Thymine and its derivatives were not converted to other pyrimidine compounds by the wild type organism. These compounds did not satisfy the pyrimidine requirement of the mutants; no growth resulted when the mutants were inoculated into minimal medium supplemented with thymine or its derivatives. These results would indicate that there is no inter-

relation between thymine and cytosine-uracil metabolism in this organism. However, methyl cytosine was readily converted to thymine by the wild type organism.

The metabolism of cytosine and its derivatives is closely related to uracil metabolism in this organism. Cytosine is converted to uracil; uracil, uridine, and uridylic acid are produced from cytidine. Growth on cytidylic acid results in uracil production. Cytidylic acid does not appear to be deaminated to uridylic acid, judging from chromatographic studies. It is perhaps either hydrolyzed to cytidine, then deaminated to uridine, and hydrolyzed to uracil, or it is broken down to cytosine and then deaminated to form uracil. Since cytidine is converted to uridine and uracil, and both uridine and uridylic acid are converted to uracil by growing cultures, both pathways probably occur. It is interesting to note that although the cytosine compounds are readily converted to uracil compounds, the converse could not be demonstrated. It would be expected that these reactions would be reversible. Failure to observe conversion of uracil compounds to cytosine or its derivatives could result if the equilibrium of the reaction lies preponderantly in the direction of formation of uracil and related compounds.

Failure to isolate orotic acid riboside either from the supernatant or from the cells could indicate that the metabolism of orotic acid does not proceed via that intermediate in this organism. However, it could

have been produced in such small amounts that it could not be detected, particularly if it were rapidly converted to other intermediates.

Uridylic Acid Accumulation

It is interesting to note that uridylic acid is the sole pyrimidine compound which accumulated in the cells in the presence of pyrimidine supplements. No accumulation occurred when the cells were grown in a peptonized milk medium in the absence of pyrimidine supplements. This accumulation of uridylic acid would seem to indicate that biosynthetic reactions leading up to uridylic acid formation occur rather rapidly and hence the intermediate products, uracil and uradine, do not accumulate in the cells. The accumulation of uridylic acid may indicate that the biosynthetic reactions which lie beyond uridylic acid take place at a much slower rate. Thus when an excess of pyrimidine is present, uridylic acid might accumulate. Since no uridylic acid was observed when the organism was grown in the absence of added pyrimidine compounds, it would appear that under normal conditions, uridylic acid is formed in a very small amount which is utilized so rapidly for biosynthesis that its presence can not be demonstrated.

Resting Cells

Although the conversion of aspartic acid, ureidosuccinic acid, and

orotic acid to uracil could be demonstrated with growing cultures, these same reactions did not occur with resting cells. This observation would indicate that the biosynthesis of uracil in A. aerogenes involves a complicated system which could not operate under the conditions used. Since some reactions which can not be demonstrated with resting cells are nevertheless observable with cell-free extracts, the biosynthesis of uracil was also attempted using cell-free extracts.

Cell-free Extracts

The results obtained with cell-free extracts are difficult to interpret. Uracil, and in some cases, orotic acid and uridine, were found in the control mixtures as well as in the experimental flasks. It is debatable whether the amounts of uracil, orotic acid, and uridine were significantly greater in the experimental mixtures than in the controls. However, the fact that in all cases, the amounts of uracil, orotic acid and uridine found in the experimental flasks exceeded that in the endogenous flasks can not be ignored.

There was always more uracil produced from ureidosuccinic acid than from orotic acid. This could have been due to the higher substrate concentrations of ureidosuccinate used.

If the amounts of uracil, orotic acid, and uridine produced above endogenous values are significant, the low values must be due to an

equilibrium which lies preponderantly in the direction of catabolism rather than biosynthesis. It may be that the cell-free extracts were catalyzing the formation of these compounds from orotate and ureidosuccinate, but that the equilibrium was such that only small amounts were formed.

It is difficult to draw unequivocal conclusions from these data. At such low levels of conversion, the effect of traces of substrate in the extract as well as breakdown of nucleoprotein present in the extract becomes proportionately greater. In order to state definitely that these results are significant, perhaps a larger number of experiments should be run. Another approach would be to study the production of orotic acid, uracil, and uridine by cell-free extracts using labeled ureidosuccinate and orotate.

SUMMARY

1. The applicability of mutants as a tool in analyzing the reaction sequences of pyrimidine biosynthesis in bacteria has been demonstrated.

2. Recombination did not appear to occur in Aerobacter aerogenes NRRL #199.

3. Four distinct types of pyrimidine auxotrophs of A. aerogenes have been produced. Type A mutants can grow only on uracil, cytosine or the corresponding nucleosides or nucleotides. Type B mutants can utilize orotate in addition to these compounds. Type C mutants respond to ureidosuccinate, orotate, or uracil. The type D mutants utilize these compounds as well as aspartate. The latter type of mutants also respond slightly to oxalacetate.

4. The following reaction sequence leading to the formation of uracil appears to occur in this organism:

Oxalacetate → Aspartate → Ureidosuccinate → Orotate → Uracil.

5. Variations in temperature had no effect on the mutant pyrimidine requirement.

6. The response to the postulated uracil precursors occurred after a lag of 14-16 hours, and reached a maximum after 20-24 hours incubation.

7. Uracil can serve as a sole source of nitrogen for this organism but not as a sole source of carbon and energy.

8. The accumulation of intermediates by the mutants could not be demonstrated.

9. Various pyrimidine compounds accumulated in the supernatant when growing cultures of the wild type or mutant organisms were grown in minimal medium supplemented with different pyrimidine compounds or postulated precursors. The significance of these reactions was discussed.

10. The conversion of orotate to uracil, or the conversion of ureidosuccinate to orotate and uracil could not be demonstrated with resting cells.

11. The presence of orotic acid riboside could not be demonstrated.

12. Uridylic acid was the sole pyrimidine compound accumulated in the cells of this organism when grown in the presence of pyrimidine supplements.

13. Small amounts of uracil and uridine were shown to be produced by cell-free extracts of A. aerogenes in the presence of orotate; small amounts of orotic acid, uracil, and uridine were produced in the presence of ureidosuccinate. The significance of these results was discussed.

14. Mutants of miscellaneous types were also produced. Some of these mutants responded to purines and to an enzymatic (desoxyribonuclease) digest of calf thymus. Others responded only to the desoxyribonucleic acid digest. These latter mutants may require a dinucleotide or more complex molecule for growth, since they did not respond to any of the desoxyribose mononucleotides.

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